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REVIEW

# Protected Areas for Biodiversity Conservation and Environment Education in the Philippines

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The Philippines holds the distinction of having enormous biodiversity with the highest density of endemic species but has the problems of very fast decline in old-growth forests and the highest number of endangered mammal and bird faunas in the world. Among the recorded 53,577 species in the country are 512 unique species of land birds, mammals, reptiles and amphibians with 43-73% endemicity. This biodiversity is seriously threatened by habitat destruction due to the expansion of human populations and activities. Loss of biodiversity impairs ecosystem functions and results in floods, drought, erosion, pests and diseases, low productivity, and food shortages, with serious socioeconomic consequences. To arrest the loss of biodiversity, *in situ* conservation is imperative and the remaining natural habitats and biodiversity must be protected. But the 73 million Filipinos in 1997 demand more land, water, biological resources, and income. Most Filipinos are unaware about the country's biodiversity and the imperative for conservation. Environment education for the general public is essential, and nature recreation and ecotourism can be effective means towards 'greening' the minds of citizens. The National Integrated Protected Areas System includes 290 sites occupying about four million hectares (about 13% of the country's total land area), mostly in the remaining forests, but increasingly more in marine ecosystems in the country. This paper provides information about the biodiversity in the protected areas, their ecotourism status or potential, and the threats to them. Many protected areas have been exploited for products and energy, only some provide for ecotourism, and only a few are actually protected. Some accessible areas should be funded and managed more effectively for ecotourism and public education, but others must be left alone and actively protected. Encounters with nature engender pride in the national heritage, generates responsible citizen action, and helps ensure biodiversity conservation.

Key words: *Sierra Madre, Isarog, Panay-Negros, Apo, Tubbataha*

The Philippines has been described as the "Galapagos Islands times ten" because of its exceptional array of biological diversity, resulting from its ancient geological history, Ice Age history, elevational range, and climate (Heaney & Regalado 1998). DENR-UNEP (1997) estimates that the Philippines has about 53,577 species of plants, animals, and microorganisms, but the counts may still be higher. Some 512 species of terrestrial vertebrates exist only in the Philippines and nowhere else in the world (43-73% endemicity, highest among amphibians and reptiles), compared with 725 unique species in Brazil, which is 28 times larger in area (Heaney & Regalado 1998). For example, there are 556 species of birds in the Philippines, belonging to 19 orders and 73 families, and including six endemic genera and 172-177 endemic species (Tabaranza & Mallari 1997).

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Biodiversity is rapidly diminishing in forest, upland, and coastal environments in the Philippines and throughout the world (Wilson 1988, UNEP 1991, WCSP 1997, DENR-UNEP 1997, Heaney & Regalado 1998). The Philippines is recognized worldwide as having: (i) enormous faunal and floral diversity, with the highest density of endemic species; (ii) the highest deforestation rate, a decline in old-growth forests from 70% to 7% cover in less than 100 years; and (iii) the most endangered mammal and bird faunas in the world, with 40 endangered endemic birds (Oliver & Heaney 1997, Heaney & Regalado 1998).

The primary cause of loss of biodiversity is not direct human exploitation or malevolence, but the habitat destruction that inevitably results from the expansion of human populations and human activities (Ehrlich 1988). Only about 872,000 ha of old-growth lowland forest and about one million hectares of montane forest remain in the Philippines. Loss of biodiversity exerts its primary impact on society through the impairment of free ecosystem services such as photosynthesis, pollination, pest control, nutrient cycling, food supply, and control of flood, drought, and erosion (Ehrlich 1988, Heaney & Regalado 1998).

The Philippines has been identified by Birdlife International as the most important country worldwide for biodiversity conservation (Tabaranza & Mallari 1997). *In situ* conservation is imperative, that is, natural habitats and biodiversity must be preserved and protected so that ecosystem services continue. But arresting the loss of diversity is extremely difficult. The increasing population (73 million Filipinos in 1997) requires more land, water, biological resources, and income. The majority of the Filipinos, even within government and other policy- and decision-making institutions, are unaware of the state of the country's biodiversity and the need for conservation. Thus, conservation efforts must include, and can only succeed with, environment education for the general public. People have to recognize their own responsibility for biodiversity loss before they can act to protect and conserve.

Formal environment education is advancing in the Philippines, but is still far from achieving its holistic and interdisciplinary objectives (EENP 1996). Environment education may also be integrated into various recreation and culture activities such as visiting protected areas and other biodiversity exhibits such as museums, zoos, and botanical gardens (Bagarinao 1998). Awareness of the natural heritage generates pride and curiosity; visits and first-hand experience generate awe, pleasure, and hopefully, protectiveness towards nature. The Philippines can learn a few lessons from fellow Asian countries, which make significant revenues from ecotourism and from well maintained and well funded nature parks. More importantly, ecotourism can contribute to the greening of the minds of the citizens.

This paper summarizes scattered and recently published information about the protected areas in the Philippines, their characteristic biodiversity and other natural attributes, and their ecotourism status or potential. The purpose is to inform more Filipinos about an important part of the national heritage (fauna, flora, ecosystems), encourage appropriate ecotourism in selected parks, and engender protectiveness toward nature, so that the citizens themselves act in ways that ensure the conservation of biodiversity. This paper expands the main ideas of Bagarinao (1998) for local environment educators, and resounds the call to action to all sectors of society to do something now, "lest we forever know our precious species only as part of a book called the Red Data Book under the category EXTINCT" (WCSP 1997), or as the "vanishing treasures of the Philippine rain forests" (Heaney & Regalado 1998).

## National Parks and Other Protected Areas

National parks, as defined by the International Union for the Conservation of Nature and Natural Resources (IUCN), are relatively large natural areas not materially altered by human activity, where extractive resource uses are not allowed, and which are established to protect outstanding natural and scenic areas of national or international significance for scientific, educational, and recreational uses. The American government in the Philippines established forest reserves as early as 1910 and in 1932, the Philippine Legislature passed Act No. 3915 establishing national parks in the Philippines (DENR-UNEP 1997). Mt. Makiling in Laguna was declared a national park by the environmentalist Governor-General Theodore Roosevelt on 23 Feb 1933 and in 1963 was made part of the University of the Philippines at Los Baños.

By the 1980s, the Philippines had 65 national parks, including ten historical sites, several hot springs or recreational lakes and waterfalls, and many others that did not meet IUCN standards (Figure 1; DENR-UNEP 1997, Bagarinao 1998). Efforts toward proper management of national parks have been hampered by various land and water use conflicts, and by the lack of political will, funds, trained personnel, and scientific research. Many national parks have been exploited rather than protected. For example, geothermal power is extracted in Tiwi Hot Springs and Mt. Apo, and logging is rampant in Bicol National Park and other parks. These and other alterations of the natural landscape violate the fundamental concept of national parks.

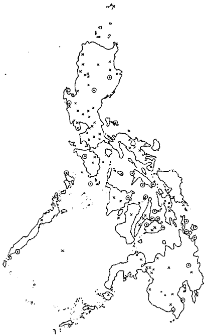

**Figure 1.** Distribution of the protected areas in the Philippines, most of which are described in the text. The protected areas system includes all localities that had been proclaimed or designated by law, presidential decree or proclamation, executive order, or administrative order, as national park, marine reserve, protected landscape and seascape (all marked x), game refuge and bird sanctuary, wilderness area (both marked + ), ⊙ watershed reserve, mangrove reserve, fish sanctuary, tourist zones (not marked).

The National Integrated Protected Areas System (NIPAS) Law was passed in 1992 to protect the remaining areas of high biodiversity in the Philippines and to zone these into protected cores and multiple-use surroundings where resources are managed on a sustainable basis by the local communities (DENR-UNEP 1997). The protected areas system includes all areas or islands that had been proclaimed, designated, or set aside pursuant to a law, presidential decree or proclamation, executive order or administrative order as national park, game refuge and bird sanctuary, wilderness area, nature reserve, watershed reserve, mangrove reserve, marine reserve, wildlife sanctuary, fish sanctuary, natural and historical landmark, and protected landscape and seascape. As of 1997, 290 protected areas are listed, occupying four million hectares, largest in Region IV (1,340,000 ha) and Region II (658,000 ha) (DENR-UNEP 1997). Thus, protected areas now comprise 13% of the Philippines' land area, and 7% of land-plus-coastal-waters.

To protect the remaining biodiversity, at least one large pristine area containing a whole complement of endemic species must be protected in each of the major faunal regions in the country. The Philippines consists of eight major faunal regions corresponding to larger islands that existed 12,000-18,000 years ago, during the Pleistocene Ice Age, when the sea water level was 120 m lower than today's: Batanes-Babuyan;

Greater Luzon including Marinduque, Catanduanes, and Polilio; Greater Palawan; Greater Mindoro; Greater Panay-Negros including Guimaras, Cebu, Masbate, and Ticao; Sibuyan; Greater Mindanao including Bohol, Leyte, Samar, Dinagat, Siargao, and Basilan; and Greater Sulu including Jolo and Tawi–tawi (Heaney 1986, 1993, Oliver & Heaney 1997). The distribution limits of endemic terrestrial vertebrates that could not fly nor swim across seas correspond to these Ice Age islands (Heaney 1993).

The Philippines has 180 species of terrestrial mammals (115 endemic), with 44 species (37 endemic) endangered to some degree and occurring in one or the other of the faunal regions. An index of faunal endangerment ranks the Greater Negros-Panay region as the most critical, followed by Mindoro and Greater Luzon (Heaney 1993, Oliver & Heaney 1997). To adequately protect entire local faunas, protected areas must (i) include as much primary forest as possible, (ii) be located on the largest island of a given faunal region, (iii) include the widest elevational range possible, from lowland rain forest to mossy forest, and (iv) be large enough to sustain viable populations of species that naturally occur at low densities; they should be designed to include a fully protected core area of primary forest surrounded by concentric buffer zones of regenerating secondary forest and managed forest plantations (Heaney 1993, Rickart 1993). These recommendations may have been considered in the selection of several of the priority sites under the NIPAS program, but there are glaring omissions for the Greater Negros-Panay faunal region.

The Protected Areas and Wildlife Bureau (PAWB) of the Department of Environment and Natural Resources (DENR) was established in 1987 to manage, protect, sustainably develop, and rehabilitate protected areas to ensure the conservation of biodiversity for national economic and social development. PAWB collaborates with non-government organizations in many aspects of environment education and wildlife conservation. PAWB and the Asian Wetlands Bureau together established the Olango Island Wildlife Reserve. Under the Debt-for-Nature-Swap Program, PAWB, the World Wildlife Fund Philippines, and the Haribon Foundation together manage the El Nido Marine Reserve, St. Paul Subterranean River National Park, and the Mt. Pulog National Park. In 1995, the Global Environmental Facility provided US$20 million through the World Bank for the Conservation of Priority Protected Areas Program, wherein PAWB and a consortium of non-government organizations implement the NIPAS Act at ten priority sites: Batanes Islands, Northern Sierra Madre, Subic-Bataan, Apo Reef, Mt. Canlaon, Siargao Island, Agusan Marsh, Mt. Kitanglad, Mt. Apo, and Turtle Islands (Magdaraog 1998). Similarly, PAWB and the European Union implement the National Integrated Protected Areas Project at eight other priority sites: Mt. Pulog, Mt. Isarog, Mindoro, Sibuyan Island, Coron Island, El Nido, Malampaya Sound, and Mt. Malindang.

Many new NIPAS sites, including seven of the 18 priority sites, are marine ecosystems and wetlands, and this direction is appropriate for the archipelagic Philippines. Several marine parks and sanctuaries have been established through the efforts of universities. Dr. Angel Alcala of Silliman University pioneered the establishment of marine reserves in Sumilon Island and extended it to Apo, Pamilacan, Balicasag, and similar island reefs in the Visayas (Alcala 1988, White 1989, Russ & Alcala 1996). The Taklong National Marine Reserve in Guimaras, declared in 1990, started out as a marine field station of the University of the Philippines-Visayas. To focus more attention and funds on marine biodiversity and conservation, the DENR under former Secretary Alcala launched the Coastal Environment Program (CEP) in 1993, wherein 14 coastal sites around the country were assessed for resources and managed with the local community for sustained economic benefits. Some of the CEP sites have been declared as protected areas: Palaui Island Protected Landscape and Seascape (PLS) in Cagayan, Masinloc-Oyon Bay Marine Reserve in Zambales, Sagay PLS in Negros Occidental, Guian PLS in Eastern Samar, and Pujada Bay PLS in Davao Oriental.

Some national parks have long been popular in tourism and thus accessible to Filipinos and foreigners, for example, Mt. Makiling, Mt. Arayat, Mt. Banahaw, Mayon Volcano, the Hundred Islands, Pagsanjan Falls, and Taal Lake and Volcano. Postcards, posters, tourist brochures, newspaper and magazine articles, and some books are available about the more popular destinations. However, many more protected areas are unknown and hardly accessible and contribute little to conservation, science, environment education, recreation, or livelihood through ecotourism. The situation must change for the better. For this, information dissemination among the general public is crucial.

## Protected Areas in Luzon

Proclaimed only in 1994, the Batanes Islands Protected Seascape and Landscape includes both marine and terrestrial ecosystems (213,576 ha) and are an important flyway for migratory birds. The endemic birds include the rail *Rallina eurizonoides alvarezi* and the green pigeon *Treron formosae filipina.* The severely endangered Ryukyu woolly flying fox *Pteropus dasmyllus* has been reported in the forests of Batanes, Dalupiri and Fuga islands (Heaney et al. 1997). Mt. Iraya (1,008 m high) and Mt. Matarem are centers of plant biodiversity, which includes the giant reed *Miscanthus sinensis condensatus* (DENR-UNEP 1997).

Mt. Data and Mt. Pulog in the Cordilleras have mountain lakes, deep ravines, forests of the Benguet pine or 'saleng' *Pinus insularis*, and a diverse wildlife including cloud rats (DENR-UNEP 1997). Both are under threat from forest fires and vegetable and flower gardening. Mt. Pulog (2,922 m high, second highest in the country) is an important watershed, a center of plant biodiversity, and has 33 endemic out of 94 species of birds. The slender-tailed Northern Luzon giant cloud rat *Phloemys pallidus* and the Luzon bushy-tailed cloud rat *Crateromys schadenbergi* are unique to the highlands of northern Luzon and important to the tribes in the Cordillera region as food and as sources of hide for bags, belts, and hats (Pasicolan 1993, Heaney et al. 1997). The rats *Batomys granti* and *Rhynchomys soricoides* are found only on Mt. Data and several other rat species are found only in Benguet, Ifugao, and Mountain Province (Heaney et al. 1987). The rodent family Muridae has 61 species in the Philippines, with 20 species endemic to Luzon (Heaney 1993, Rickart 1993).

The internationally known Banawe Rice Terraces (up to 1,500 m high and 2,000 years old) have not been declared a national park despite its cultural and biological significance. Thankfully, the Ifugao rice terraces, including those in Banawe, have recently been declared a UNESCO World Heritage Site. Protection is urgently needed as the rice terraces are eroded and Banawe is degraded by commercial tourism. Recently, the Department of Tourism made the Ifugao rice terraces the focus of conservation and ecotourism.

The Northern Sierra Madre Natural Park has the largest block of old-growth primary lowland evergreen rain forest (247,861 ha) in the country, as well as five other distinct forest types (DENR-UNEP 1997). The vegetation includes the endemic dipterocarps *Shorea* and *Hopea* species, orchids *Dendrobium aclinia* and *Eulophia dentata*, the legume tree *Milletia longipes*, and the *citrus Swinglea glutinosa.* A unique frog, *Platymantis pygmaeus*, only 1.5 cm long, lives in the Sierra Madres (Alcala et al. 1998), and so do the Sierra Madre shrew mouse *Crunomys fallax*, the Luzon-endemic *Apomys datae* and *A. sacobianus* and other rodents (Mallari & Jensen 1993). Also in the forest are the widespread palm civet *Paradoxurus hermaphroditus*, Malay civet *Viverra tangalunga*, monkey *Macaca fascicularis*, and the endemic Philippine brown deer *Cervus mariannus* and Philippine warty pig *Sus philippensis.* Among the resident bats are the endangered golden-crowned flying fox *Acerodon jubatus* (largest bat in the world by weight), the white-winged flying fox *Pteropus leucopterus*, and the dwarf fruit bat *Haplonycteris fischeri*, Philippine endemics absent from the Palawan faunal region (Mallari & Jensen 1993, Heaney et al. 1997).

The Sierra Madres are one of the 221 Endemic Bird Areas (nine in the Philippines alone) globally targeted for conservation (Mallari & Jensen 1993). The Sierra Madres have 241 species of birds (200 breeding and 41 migratory), including 78 Philippine endemics, 19 Luzon endemics, and 28 globally threatened forest birds. These mountains harbor some of the last 30 breeding pairs of the endangered Philippine eagle *Pithecophaga jeffreyi* (Lewis 1986) and the only remaining population of the flame-breasted fruit dove *Ptilinopus marchei*, which is considered as the most beautiful of the 33 species of doves and pigeons (16 of them endemic) in the Philippines (Heaney & Regalado 1998). The Isabela oriole *Oriolus isabellae* has not been seen since 1967 and is likely to be the first of Sierra Madres' bird species to face global extinction (Mallari & Jensen

1993). Logging and slash-and-burn farming threaten the entire Sierra Madre south to Aurora and Quezon provinces; from four million hectares of primary lowland forests in the 1930s, only 685,000 ha remain. The indigenous Agta people are the crucial agents in the protection of the Sierra Madres (Mallari & Jensen 1993).

The Sierra Madre Natural Park also includes 71,652 ha of coral reefs, seagrass beds, and mangroves (DENR-UNEP 1997). The green sea turtle *Chelonia mydas* and the hawksbill *Eretmochelys imbricata* are sighted in the park, the manatee *Dugong dugong* along the coast from Isabela to Quezon, and a remnant population of the saltwater crocodile *Crocodylus porosus* in the rivers in the eastern Sierra Madre and in the Cagayan River (Mallari & Jensen 1993, WCSP 1997).

The Subic-Bataan National Park is home to sizeable populations of fruit bats, monkeys, birds, and many other animals and plants in the lowland rain forest and grasslands, and even more in the coral reefs and mangroves. The World Wildlife Fund and other international agencies conduct research and training in biodiversity and resources management in Subic, which is now a popular ecotourism destination. Visitors can go right into the Subic Bay Forest Reserve, where the dominant trees are *Shorea contorta, Parkia roxburghii, Glochidium puberum, Ervatamia mucronata*, and the bamboo *Schizostachyum lumampao*, where the flat-headed bats *Tylonycteris pachypus* and *T. robustula* roost (DENR-UNEP 1997). The Subic forest was blanketed in thick ash from the Mt. Pinatubo eruption in 1991, but was not permanently harmed.

Mt. Pinatubo (1,780 m high) itself is feared to have had a local mass extinction that wiped out its 60 endemic species of plants, including the lipstick plant *Medinilla negrito*, the oak *Quercus pinatubensis*, the legume *Dalbergia pinatubensis*, and the orchids *Bulbophyllum zambalense* and *Dendrochilum foxworthyi* (Madulid 1992). The Aetas, the indigenous people of Zambales, were evacuated out of Mt. Pinatubo, but the forest animals were not so lucky. Nevertheless, mountaineers who have been to Pinatubo recently report: "... a lush mountain covered in greenery, fed by an idyllic stream of cool medicinal water. Grasses were abundant, shrubs and mosses lined the walls carved out by the water, and in some places, saplings had already taken root, competing to be the first trees of the next generation... The flying animals were first to colonize this virgin territory... The first animals we noticed were the insects, specifically the winged ants..." (J. Rufino, Philippine Daily Inquirer, 23 May 1999).

Limestone caves are found all over the Philippines. They need special protection because they are interesting sites for ecotourism, they are rich sources of marble, stalactites, stalagmites, and guano, and they are fragile habitats for highly specialized and often endemic plants and animals. The characteristic vegetation consists of figs *Ficus* spp., molave *Vitex parviflora*, ferns, and mosses; the wildlife includes snakes, birds such as swiftlets *Collocalia spp.*, and insectivorous bats such as the Asian ghost bat *Megaderma spasma* (Heaney & Regalado 1998). The Callao Caves, now part of the Peñablanca Protected Landscape and Seascape in Cagayan, are noted for the millions of bats that come out around sunset; 51 species of birds and 29 mammals are found in and around these caves (DENR-UNEP 1997). Central Luzon has many cave systems, among them the Minalungao Cave in Nueva Ecija and the Apaan Cave in Zambales. In the Angat Limestone Formation in Bulacan's Biak-na-Bato National Park is Madlum Cave, and nearby are the historical Aguinaldo Cave and the Bahay Paniki Cave. The Pamitinan Protected Landscape in Rizal is 600 ha of cave ecosystem threatened by rock-quarrying in nearby mountains.

Mt. Makiling, a 1,050-m high inactive volcano, is the most accessible natural forest from Manila and the best studied forest ecosystem in the country, due to the students and scientists at the University of the Philippines-Los Baños (Caldecott 1993). Mt. Makiling has 2,038 species of flowering plants, including endemic dipterocarps and the beautiful jade vine *Strongylodon macrobotrys* first seen in 1854 by a United States Exploring Expedition (DENR-UNEP 1997, Heaney & Regalado 1998). Faunal inventories in Mt. Makiling include 132 species of birds, 23 bats, 23 amphibians, 29 lizards, 38 snakes, 5 skinks, a crocodile, a turtle, and a shrew, many of them endemic and threatened (Mendoza 1987, Catibog-Sinha 1987, Ingle 1992). The forest-dependent endemic bear-faced bat *Ptenochirus jagori* comprises 74% of bats caught by mist nets (Ingle 1992). Also in Mt. Makiling are the endangered golden-crowned flying fox, white-winged flying fox, and Philippine dwarf fruit bat (Ingle 1992, Heaney et al. 1997).

Taal Lake and Volcano, Pagsanjan Falls, and several other park destinations in southern Luzon have well established tourist services, but the bus drivers, guides, boatmen, and hotel personnel hardly provide information about the geology and natural history of these areas. Such information is available but not widely learned nor disseminated. For example, much can be learned from Thomas Hargrove's (1991) intriguing book about Taal Lake and Volcano and its freshwater-adapted sea life, including the sardine *Sardinella tawilis* and the sea snake *Hydrophis semperi*. Taal Lake (and Naujan Lake in Mindoro) used to have many migratory fishes such as mullets, snappers, jacks, and milkfish, but these have now been replaced by the introduced tilapia (Herre 1958, Mercene & Alzona 1984).

Whereas northern Luzon still has relatively large tracts of forests in the Sierra Madres and the Cordilleras, the south has been mostly denuded and the majority of species unique to this region are now seriously threatened with extinction. Southern Luzon has been identified by Birdlife International as one of the world's ten highest priority conservation areas in terms of both degrees of endemism and threat (Oliver & Heaney 1997). Logging and human encroachment have degraded Quezon National Park and Bicol National Park. The environmental impact assessment required for the construction of the Quezon Coal-Fired Power Plant on Pagbilao Grande Island and the exploration for geothermal power on Mt. Labo in Camarines Norte produced data on plant diversity (Gruezo & Badayos 1996, Gruezo 1998) that ironically will be compromised by the two projects. The Caramoan and Libmanan Caves in Camarines Sur need assessment and protection. Gray's monitor lizard *Varanus grayi* can be found in *Ficus-Vitex* forests on limestone cliffs and lowland dipterocarp forests, only in the Bicol peninsula and Catanduanes (WCSP 1997).

Biodiversity remains high on Mt. Isarog (1,966 m high), home to the the indigenous Agta, and the all-important watershed of Naga City. The mountain has a wide range of habitats from primary lowland dipterocarp forest with ravines and waterfalls, to lower montane forest with pitcher plants and orchids, to upper montane or mossy forest (Heaney & Regalado 1998). Mt. Isarog has many species of endemic wildlife, including various amphibians and reptiles (Alcala 1986), and more than 135 species of birds, 116 of which breed on the mountain (Goodman & Gonzales 1990). Among the 45 mammal species on the mountain are several unique cloud rats, including the Southern Luzon giant cloud rat *Phloemys cumingi*, the Isarog shrew mouse *Archboldomys luzonensis*, Isarog striped shrew rat *Chrotomys gonzalesi*, and the Isarog tweezer-beaked shrew rat *Rhynchomys isarogensis* (Heaney et al. 1997). In the lower montane forest, the harpy fruit bat *Harpionycteris whiteheadi* roosts above the climbing pandan *Freycinetia*, eats its fleshy bracts, and pollinates it in the process. The Luzon-endemic monotypic fruit bats *Haplonycteris fischeri* and *Otopteropus cartilagonodus* also live on Mt. Isarog (Utzurrum 1992, Heaney & Regalado 1998).

Further south in the Bicol peninsula are Mt. Iriga and other volcanic peaks looming over Lakes Buhi, Bato, and Manapao, the home of the endemic 'sinarapan' goby *Mistichthys luzonensis* (adult size 12 mm), long threatened by overfishing and predation by the introduced tilapia (Soliman 1994). Mt. Mayon with the picture-perfect cone is an active volcano, scorched and nearly barren at high elevation, but covered with tall grasses, tree ferns, short trees, and various epiphytic plants on the lower slopes. Hoyop-hoyopan Cave is well visited and no longer has resident bats, but has a window with a nice view of Mt. Mayon and its fiery eruptions. Bulusan Lake and Volcano in Sorsogon are not as popular as Mayon Volcano or Tiwi Hot Springs, but the area is very beautiful, advertised as "like a Swiss alpine lake." Whale sharks *Rhincodon typus* frequent the coasts of Donsol, Sorsogon and have attracted tourists. Nearby Catanduanes Island has about 239 species of birds and 35 species of mammals, mostly similar to those in Luzon (Gonzales 1983, Heaney et al. 1997).

## Protected Areas in Mindoro and Palawan

Mindoro and Palawan are geologically the oldest islands in the Philippine archipelago, having been originally parts of the Asian mainland (Heaney & Regalado 1998). The Palawan faunal region is actually part of the Sundaic faunal region, along with Borneo, Sumatra, and Java, and thus has many species (peacock pheasant, bear cat, otters, mouse deer, etc.) found nowhere else in the Philippines, but widely distributed elsewhere in southeast Asia.

The whole of Mindoro is one of the world's ten highest priority conservation areas (Oliver & Heaney 1997). Mindoro is best known for the endangered dwarf water buffalo or 'tamaraw' *Bubalus mindorensis*, discovered in 1888, the largest mammal endemic to the Philippines, 1 m tall at the shoulder and 200 kg when fully grown. The tamaraw once occurred throughout Mindoro from sea level to 2,000 m high in the mountains, but now occur only in a few remote mountains at high elevations. They numbered 10,000 at the turn of century, but only 1,000 in the late 1940s after loggers and farmers settled in Mindoro when anti-malaria medicine became available. Hunters came with automatic weapons in the 1960-1970s, and the tamaraw population shrank to 175 in 1983. Mt. Iglit-Baco National Park in central Mindoro, with 75,445 ha of natural grasslands and dipterocarp forests, was set aside as tamaraw habitat in 1970 and later became an ASEAN Heritage Site. A tamaraw conservation program was started in 1979 and tamaraw biology was studied for management and captive breeding, but these efforts have not helped the species (Heaney et al. 1997).

The mountains in northwestern Mindoro has forests of the 'tapulao' pine *Pinus merkusii*, a species also found in Zambales, Burma, and the Himalayas (Heaney & Regalado 1998). Mt. Halcon (2,586 m high) in the Mangyan Heritage Park in central Mindoro is the ancestral domain of the Mangyan people and is a center of plant biodiversity (DENR-UNEP 1997). The amphibians and reptiles of Mindoro are described by Alcala (1986). The shrew *Crocidura mindorus* and climbing rat *Anonymomys mindorensis* are known only from about 1,900 m high on Mt. Halcon (Heaney et al. 1997). These and the Mindoro forest mouse *Apomys gracilirostris*, the rat *Rattus mindorensis*, and the shrew *Crocidura grayi* are threatened by habitat loss. The hairy-tailed cloud rat *Crateromys paulus* from Ilin Island south of Mindoro may be extinct. The endemic scops-owl *Otus mindorensis*, the black-hooded coucal *Centropus steeri*, the hornbill *Penelopides mindorensis*, the imperial pigeon *Ducula mindorensis*, and the Mindoro *bleeding-heart* pigeon *Gallicolumba platenae* are all endangered (Tabaranza & Mallari 1997).

At the north coast of Mindoro is Puerto Galera, a UNESCO Man and the Biosphere Reserve with coral reefs and beaches now much affected by tourism. Gonzales (1993) found 76 species of birds in six habitat types in Puerto Galera, including the Mindoro-endemic scarlet-collared flowerpecker *Dicaeum retrocinctum*. Off the southwest coast of Mindoro is the Apo Reef Marine Natural Park with 29 ha of land and 15,763 ha of shallow reefs with 33-46% coral cover. Here live the hawksbill and green sea turtles, at least 280 species of fish, and 24 birds including the endangered Nicobar pigeon *Caloenas nicobarica* (DENR-UNEP 1997).

Naujan Lake, the third largest lake in the country, is a national park and the type locality of the endemic freshwater crocodile *Crocodylus mindorensis* and the carp *Puntius hemictenus* (not recently recorded). The lake is an important staging and wintering area for large numbers of ducks, the endangered Chinese egret *Egretta eulophotes*, and other wetland birds (Davies 1992). *Crocodylus mindorensis* used to be widely distributed in the freshwater marshes and river tributaries in Luzon, Mindoro, Busuanga, Masbate, Negros, Samar, Mindanao, and Jolo, but it is now critically endangered, with only about 100 individuals left at a few sites in these islands, and about the same number now in captivity (Ortega et al. 1993).

Palawan is probably the most popular ecotourism destination in the Philippines today. The entire Palawan Island was gazetted as a game refuge and wildlife sanctuary in 1967, as a mangrove swamp forest reserve in 1981, as a flora and fauna and watershed reservation in 1982, and also as a Man and the Biosphere Reserve. Palawan and largely pristine Coron Island are both centers of plant biodiversity, with more than 2,000 species of flowering plants (DENR-UNEP 1997). Ulugan Bay and Malampaya Sound have old-growth mangrove forests and rice marine resources. The St. Paul Underground River in Puerto Princesa and the El Nido Marine Reserve both have caves, fantastic rock formations, and limestone orests with monkeys, bats, and many species of birds. Lake Manguao, the only lake in Palawan, has three ndemic species of carps (not recently collected) and is urrounded by high-quality rain forest (Davies 1992).

Calauit Island off Busuanga was declared a Game Reserve and Wild Animal Sanctuary in 1976 to protect the eight species of introduced herbivorous mammals from Africa. The afforded protection has allowed not only the African mammals, but more importantly, the endemic and resident wildlife to reproduce. Some 236 species of land vertebrates have been recorded in Calauit over the last 20 years: 6 amphibians, 28 reptiles, 170 birds, and 32 mammals (Alviola 1998), including the following species, which are mostly endemic and endangered:

| | |
|---|---|
| Calamian deer | *Cervus porcinus calamianensis* |
| Mouse deer | *Tragulus napu nigricans* |
| Palawan tree squirrel | *Sundasciurus juvencus* |
| Palawan porcupine | *Hystrix pumilla* |
| Palawan bear cat | *Arctictis binturong whitei* |
| Common house shrew | *Suncus murinus* |
| Palawan tree shrew | *Anthracoceros marchei* |
| Palawan bearded pig | *Polyplectron emphanum* |
| Pangolin or scaly anteater | *Stachyris hypogrammica* |
| Palawan stink badger | *Ficedula platenae* |
| Palawan tree mouse | *Tupaia palawanensis* |
| Palawan hornbill | *Sus barbatus ahoenobarbus* |
| Palawan peacock pheasant | *Manis javanica* |
| Palawan striped babbler | *Mydaus marchei* |
| Palawan flycatcher | *Chiropodomys calamianensis* |
| Blue-naped parrot | *Tanygnathus lucionensis* |
| Philippine cockatoo | *Cacatua haematuropygia* |
| Palawan scops owl | *Otus fuliginosus* |
| Melodious babbler | *Malacopteron palawanense* |

The shrew *Tupaia palawanensis* has four subspecies throughout the different island groups in Palawan province, is common in primary and secondary forests, coconut groves, and banana plantations, and has been estimated to occur at 2-3 individuals/ha at the Iwahig Penal Farm in Puerto Princesa (Dans 1993). *Sus barbatus ahoenobarbus* of Palawan and Mindoro is closely related to the bearded pig of Borneo, Sumatra, and Malaysia, *S.b. barbatus*, which has apparently colonized Tawitawi and Sibutu (Oliver 1992). Also found in Palawan and Busuanga are the rare Oriental small-clawed otter *Amblonyx cinereus*, the Asian leopard cat *Prionailurus bengalensis*, the Asian shrew *Crocidura fuliginosa*, the endemic soft-furred mountain rat *Palawanomys furvus*, and the Palawan flying fox *Acerodon leucotis* (Heaney et al. 1997). Five different tree squirrels (*Sundasciurus* spp.) inhabit the different islands in the Palawan group; the montane tree squirrel *S. rabori* is found only on Mt. Mantalingajan on Palawan Island (Heaney et al. 1987).

The Palawan faunal region is also very rich in aquatic species. The saltwater crocodile can still be found in 44 rivers in Palawan, Balabac, Culion, and Busuanga, and are being propagated in captivity at the Crocodile Farming Institute in Irawan, Puerto Princesa (Ortega et al. 1993). Dugongs still live in Palawan waters in small numbers: fishermen report 11 dugong sightings and 4 incidental captures and eventual slaughter each year (Leatherwood et al. 1992, Trono et al. 1993). The green sea turtle, the hawksbill, and the olive ridley *Lepidochelys olivaceus*, as well as the giant clams *Tridacna squamosa*, *T. crocea*, *T. gigas*, *T. derasa*, *T. maxima*, *Hippopus porcellanus*, and *H. hippopus* still have populations in Palawan waters (WCSP 1997).

The Tubbataha Reef National Marine Park (two atolls and 33,200 ha of ocean) in the middle of the Sulu Sea was declared a marine park in 1988, and a UNESCO World Heritage Site in 1995. Only a few species of trees, shrubs, and grasses grow on the low islands. Tubbataha has some of the best coral reefs in the Philippines, with 70-80% cover from 300 species and 46 genera of corals; surveys also show 43 species of seaweeds, 5 seagrasses, 379 fishes, marine turtles, giant clams, and sea birds including brown boobies, terns, black noddies, shearwaters, frigate birds, and plovers (White & Palaganas 1991). Seaweed farms were set up by Shemberg Corporation on Tubbataha but these were dismantled in the wake of bad press (Arquiza & White1994). However, migrant fishermen continue to ply Tubbataha waters, dive tourism is expanding, and management for sustainable use is still difficult (White & Palaganas 1991).

## Protected Areas in the Visayas

Many places in Panay, Negros, and elsewhere in the Visayas have been proclaimed as protected areas (Table 1), many of them only recently, as a response to widespread environmental degradation and recent findings about high levels of biodiversity in these areas.

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## Protected Areas in the Visayas

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Table 1. Protected areas in the Visayas.

| Name | Location | Area (ha) |
|---|---|---|
| Mt. Canlaon* National Park | Negros Occ. Oriental | 24,558 |
| Sagay Protected Landscape and Seascape | Negros Occidental | 28,300 |
| Apo Island PLS | Negros Oriental | 15,792 |
| Ilog River Watershed Reserve | Negros Occidental | |
| Bago River WR | Negros Occidental | |
| Mt. Talinis* - Lake Balinsasayao WR | Negros Oriental | |
| Taklong Island National Maritime Reserve | Guimaras | 1 143 |
| Bulabog-Putian NP | Dingle & San Enrique, Iloilo | 854 |
| Sampunong Bolo Game Refuge Bird Sanctuary | Juaneza, Sara, Iloilo | 52 |
| Panagatan Marine Turtle Sanctuary | west of Antique | |
| Boracay Tourist Zone & Marine Reserve | Aklan | |
| Pan-ay River WR | Tapaz, Capiz | |
| Aklan River WR | Madalag & Libacao, Aklan | |
| Siquijor TZMR | Siquijor | |
| Sudlon NP | Cebu City | 696 |
| Central Cebu NP | Cebu, Balamban, Toledo | 15,394 |
| Mabugnaw-Mainit Springs NP | Carcar, Cebu | 53 |
| Lake Danao GRBS | Camotes, Cebu | 430 |
| Olango Island TZMR, GRBS | east of Mactan Island | 920 |
| Rajah Sikatuna NP | Bohol | 9,023 |
| Chocolate Hills Natural Monument | Carmen, Bohol | 1,776 |
| Balicasag Island TZMR | Tagbilaran, Bohol | |
| Loboc River WR | Bohol | |
| Kuapnit-Balinsasayaw NP | Baybay & Abuyog, Leyte | 364 |
| Mahagnaw NP | Burauen & La Paz, Leyte | 635 |
| Tongonan Hot Springs NP | Ormoc City | 272 |
| Mt. Sohoton NP | Basey, Samar | 840 |
| Guiuan PLS | southern Samar | 60,448 |
| Mt. Guiting-Guiting* Natural Park | Sibuyan Island, Romblon | 15,265 |

*Centers of plant biodiversity

NP national park, WR watershed reserve, GRBS game refuge and bird sanctuary, PLS protected landscape and seascape, TZMR tourist zone and marine reserve

Data from DENR-UNEP (1997)

The rugged mountains of Sibuyan Island, only 46,300 ha in area, support five unique mammals, the Sibuyan giant moss-mouse *Tarsomys sp.*, Sibuyan striped shrew rat *Chrotomys* sp., Mindoro shrew *Crocidura mindorus*, Sibuyan pygmy fruit bat *Haplonycteris sp.*, and Philippine tube-nosed fruit bat *Nyctimene rabori* (described only in 1984), and many unique flowering plants and birds (Goodman & Ingle 1993, Goodman et al. 1995, Heaney et al. 1997). In recognition of the need for both watershed and wildlife protection, Mt. Guiting-Guiting (2,052 m high) on Sibuyan was officially designated as national park in 1996 (DENR-UNEP 1997).

Mt. Canlaon (2,435 m high) is an active volcano with hot springs, gorges, waterfalls, rock formations, and high montane forests, but little lowland forest left. In addition to many dipterocarps, the mountain grows orchids, ferns, palms, rattans, pitcher plants, the giant tree 'malakawayan' *Podocarpus rumphii*, and the 'bakawan-gubat' *Carallia brachiata*. The endemic plants *Crytandra cyclopum*, *Isachne vulcanica*, and *Miscanthus depauperatus* can be found at the crater (DENR-UNEP 1997). The fauna includes many unique species — 50 species of birds, 81 butterflies, 11 mammals and numerous amphibians and reptiles – many of them endangered. The Negros green fruit dove *Ptilinopus arcanus* lived in the mid-mountain forest of Mt. Canlaon but has not been seen since 1953 and may be extinct (Tabaranza & Mallari 1997). Similarly, the forest-living Negros bleeding-heart pigeon *Gallicolumba keayi* and Negros striped babbler *Stachyris nigrorum* are critically threatened.

Cuernos de Negros or Mt. Talinis near Lake Balinsasayao in Negros Oriental also has a high diversity of plants and animals, but these are poorly known and threatened by logging, human settlements, hunting, and overharvesting. *Nyctimene rabori* is found at densities of 2-3 bats over 10 ha in the primary forests around Lake Balinsasayao, and the more abundant *Pteropus pumilis* is widely hunted (Utzurrum 1992). The Negros shrew *Crocidura negrina* is known only from six specimens taken in primary lowland and montane forest (500-1,500 m elevation) in southern Negros as recently as 1987 (Heaney et al. 1997). In the montane forests of Cuernos de Negros live 14 species of amphibians, 26 snakes, and 27 lizards, including the endemic sail-fin lizard *Hydrosaurus pustulatus* (Brown & Alcala 1961). The rare cave frog *Platymantis spelaeus*, collected from only two caves in southern Negros, has not been seen since 1981 (WCSP 1997).

Indeed the Greater Negros-Panay faunal region has many endemic forest animals. The Asian leopard cat *Prionailurus bengalensis* occurs in small numbers in agricultural habitats and forests from sea level to about 1,500 m high in Negros, Panay, and Cebu (Heaney et al. 1997). *Nyctimene rabori* roosts in the forests (never in caves) of Negros, Cebu, and Sibuyan up to 1,250 m above sea level (Heaney & Regalado 1998). The forest frog *Platymantis insulatus* inhabits the forest floor near or at the entrances of limestone caves only on South Gigante Island off northeast Panay (WCSP 1997). The Panay bushy-tailed cloud rat *Crateromys heaneyi* was discovered recently in the lowland primary and secondary forests in the western and central Panay mountains, restricted to the area (Gonzales & Kennedy 1996). In the lowland forest in northwest Panay live the Philippine nectar bat *Eonycteris robusta* and the harpy fruit bat *Harpyionycteris whiteheadi* and 23 species of birds, including the endangered Panay striped babbler *Stachyris latistriata*, green-faced parrotfinch *Erythrura viridifacies*, white-throated jungle flycatcher *Rhinomyias albigularis*, blue-naped parrot *Tanygnathus lucionensis*, and white-winged cuckoo-shrike *Coracina ostenta*.

Negros and Panay have been heavily deforested to make land for agriculture and have lost many unique wildlife species. Birds are among the most seriously threatened by habitat loss. The flame-templed babbler *Stachyris speciosa* and the Visayan flowerpecker *Dicaeum haematostictum* in Negros, Panay, and Guimaras are endangered (Tabaranza & Mallari 1997). Two of the five endemic hornbill species in the Philippines can be found only in the forests of Panay, Negros, and Masbate. In the Panay mountains, the Visayan tarictic *Penelopides panini* and the writhed-billed hornbill *Aceros waldeni* occur together at a ratio of 15-30 to 1. The global wild population of *A. waldeni* is only 50-80 pairs, with about 30-40 pairs in the central Panay mountains. A distinctive population of *Aceros waldeni* on Ticao Island is believed to be the first hornbill in the world to have gone extinct, and the other populations in Negros and Panay are likely to follow (Heaney & Regalado 1998).

The Philippine bare-backed fruit bat *Dobsonia chapmani* is the first mammal in the Philippines to have gone extinct in modern times; it roosted in caves in the lowland forests of Negros, but these caves were mined for guano and the smoke from the miners' torches suffocated the bats (Heaney & Regalado 1998). The fruit bat *Acerodon lucifer*, found only in Panay, has not been seen since 1888 and is believed to be extinct (Utzurrum 1992). The Visayan warty pig *Sus cebifrons* now survives only in a few isolated areas in Negros and Panay, where it continues to be hunted and suffers from genetic contamination through interbreeding with domestic and feral pigs; this endemic pig is already extinct in Masbate, Cebu, Siquijor, and Guimaras (Oliver 1992). Only a few hundred Visayan spotted deer *Cervus alfredi* are left in Mt. Baloy and Mt. Madjaas on Panay, and in a few scattered fragments of forests on Negros; this endemic species is already extinct in Masbate, Cebu, Siquijor, Guimaras, and Ticao (Oliver et al. 1992). To protect the Visayan warty pig and the Visayan spotted deer, a Central Panay Mountains National Park (ca. 40,000 ha) was proposed in 1987 (Oliver et al. 1992) and was declared in 1998 to include the areas around Mt. Baloy and Mt. Madjaas.

The Bulabog-Putian National Park (854 ha) in Dingle and San Enrique, Iloilo, was proclaimed in 1961. The park has extensive caves, natural water holes or 'tisok,' and limestone forests seeded by bats and inhabited by land snails. Some of the caves were used as hiding places by guerillas and others were mined for guano. At least six species of bats live in the caves: *Cynopterus brachyotis*, *Hipposideros diadema*, *Rhinolophus macrotis*, *Eonycteris spelaea*, *Taphozous melanopogon*, and *Miniopterus australis*. Snakes and swallows live on the limestone cliffs, and a poisonous spider lives deep in dark Guiso Cave. The Sampunong Bolo Bird Sanctuary in Sara, Iloilo, has a resident breeding population of the purple heron *Ardea purpurea-manilensis* coexisting with the migratory night herons *Nycticorax caledonicus* and *N. nycticorax*, and several other birds, reptiles, and bats (Ebreo 1993).

Cebu used to have 149 species of birds; of the 14 endemic species and subspecies, three species are extinct, ten species number fewer than 100 individuals each, and the four-color Cebu flowerpecker *Dicaeum quadricolor* has only four individuals alive (Rabor 1959, Heaney & Regalado 1998). Cebu is the most deforested island in the Philippines and the small forest patches in Central Cebu National Park are the only remaining habitats for the critically endangered Cebu flowerpecker, the elegant titmouse *Parus elegans visayanus*, Everett's white-eye *Zosterops everetti everetti*, Philippine hanging parakeet *Loriculus philippensis chrysonotus*, and the black shama *Copsychus cebuensis* (Magsalay 1993). Sudlon National Park and the Mabugnao-Mainit Springs also have limestone caves with bats, swifts, and swallows (DENR-UNEP 1997).

Rajah Sikatuna National Park includes the last remaining dipterocarp and mossy forests of Bohol, and is home to the Philippine tarsier *Tarsius syrichta*, the 'kagwang' or flying lemur *Cynocephalus volans*, the

Philippine warty pig *Sus philippensis*, and other land mammals shared with Leyte, Samar, and the Greater Mindanao faunal region (Heaney 1993, DENR-UNEP 1997). The bird fauna includes the Philippine trogon, black-backed coleto, green imperial pigeon, various kingfishers, and the wintering Chinese egret. The Chocolate Hills in Carmen is a karst landscape of 1,826 limestone hills (40-120 m high) that used to be shallow reefs, now 100-500 m above sea level and overgrown with grass or low shrubs that turn brown during the dry season. Long famous as a tourist attraction, the Chocolate Hills were declared a National Geological Monument only in 1997, and some hills were quarried even more recently.

The Kuapnit-Balinsasayao guano caves in Leyte and the Sohoton Natural Bridge's cathedral-like caves in Samar also teem with bats, swifts, and other wildlife (DENR-UNEP 1997). The forests of Samar and Leyte have been much reduced, but in parts still harbor civets, macaque, Philippine warty pig, Philippine brown deer and about 180 species of birds, among them the Philippine eagle and Philippine cockatoo (Heaney et al. 1997, Tabaranza & Mallari 1997).

Many of the other new protected areas in the Visayas are wetlands or coastal ecosystems — in recognition of the importance of aquatic habitats and biodiversity. The intertidal flats of Olango Island and Lake Danao's shallow waters similarly attract bird flocks and are important staging areas for migratory shore birds, particularly the endangered greenshank *Tringa guttifer*, the Asian dowitcher *Limnodromus semipalmatus*, and the Chinese egret *Egretta euphlotes* (Davies 1992). Guimaras' Taklong National Marine Reserve includes two islands and several islets fringed by coral reefs, mangroves, and sandy beaches. Once-beautiful Panagatan Cays is an incomplete atoll heavily fished by migratory fishermen using the destructive 'kayakas' (modified muro-ami) method. Many of these fishermen have settled on the three low islands and farm agar-producing seaweeds on the reef, shading the corals and the algae.

Twenty species (six families) of dophins and whales occur in Philippine waters, including the humpback whale *Megaptera novaeangliae*, minke *Balaenoptera acutorostrata*, Bryde's whale *B. edeni*, sperm whale *Physeter macrocephalus*, Blainville's beaked whale *Mesoplodon densirostris*, killer whale *Orcinus orca*, false killer *Pseudorca crassidens*, pygmy killer *Feresa attenuata*, melon-headed whale *Peponocephala electra*, and various dolphins (Leatherwood et al. 1992, Alava et al. 1993, Tan 1994). All these species are in Appendix I or II of the Convention on the International Trade of Endangered Animals (WCSP 1997). The seas between the Visayan islands and Mindanao, and particularly the Tañon Strait between Negros and Cebu, have the highest concentration of whales and dolphins in the country. Whale-watching has become a major tourist industry (in February-October) in Bais City in southeastern Negros, where spinner, spotted, and other dolphins are regularly sighted with beaked, pilot, and dwarf sperm whales.

Twelve species of whales and dolphins are harvested by incidental and directed fisheries in Pamilacan, Camiguin, Aligway, and Selinog Islands, in San Francisco, Negros Oriental, and at Brooke's Point, Palawan (Leatherwood et al. 1992, Alava et al. 1993, Tan 1994). Unregulated fishing and hunting may eliminate the Philippine populations of the rare, slow-reproducing species such as Bryde's whale, pilot whale, beaked whale, and of the dophins most frequently caught by fishing nets. Fisheries Administrative Order No. 185 issued by the Department of Agriculture in 1992 bans the taking, catching, selling, buying, possessing, transporting, and exporting of dophins and small whales of the family Delphinidae. Conservationists have sought a ban that extends to all whales and dolphins and called for the establishment of the southeastern Tañon Strait, or alternatively, the seas around Pamilacan Island, as a Marine Mammal and Wildlife Sanctuary (Alava et al. 1993, WCSP 1997). Apo Island PLS and Balicasag Island Marine Reserve are in the same general area as Pamilacan (White 1989, Russ & Alcala 1996).

## Protected Areas in Mindanao

Mindanao had 15 national parks in 1983 and several more sites were incorporated under the NIPAS Act. Almost all these areas have not been protected or managed and suffer from habitat destruction, logging, hunting, and squatting. Ecotourism has been established only for Mt. Apo, Camiguin Island, and Lake Sebu. The biodiversity on Mt. Apo has been studied since the 1970s, but only recently have efforts been made to document biodiversity elsewhere.

About 21 species of mammals are endemic to the Greater Mindanao faunal region, including the Philippine tarsier, the flying lemur, the insectivores *Podogymnura truei*, *Crocidura grandis* and the tree shrew *Urogale everetti*, the tree squirrel *Sundasciurus philippensis*, the rats *Apomys insignis* and *Crunomys melanius*, and the fruit bat *Ptenochirus minor* (Rickart 1993, Heaney 1993). The Mindanao spiny rat *Tarsomys echinatus* is known only from the lowland forests of Bukidnon and South Cotabato, and the Mindanao shrew mouse *Crunomys melanius* from primary forests below 900 m in Bukidnon, Leyte, Camiguin, Cotabato, and Davao del Norte (Heaney et al. 1997). Philippine-endemics such as *Sus philippensis*, *Cervus mariannus*, *Acerodon jubatus*, and *Eonycteris robusta*, and non-endemics like the civets and macaque, occur in both the Greater Mindanao and Greater Luzon faunal regions (Oliver 1992, Heaney 1993, Rickart 1993).

Many of the mammals and birds in Mindanao have restricted ranges and are threatened by the rapid loss of forests. The critically endangered Philippine eagle has only about 200 individuals left in the primary forests of Mindanao, Samar, Leyte, and Luzon, and has a very low reproduction rate (two eggs in two years, only one hatches) (Tabaranza & Mallari 1997). The Philippine cockatoo is also critically threatened due to hunting and has only 1,000-4,000 individuals left in forests of Mindanao, Palawan, Luzon, and the Visayas. Many endemic birds in Mindanao and elsewhere in the Philippines are listed in the Philippine Red Data Book (WCSP 1997) under the categories vulnerable, endangered, and critical:

| | |
|---|---|
| Mindanao lorikeet | *Trichoglossus johnstoniae* |
| Mindanao bleeding-heart | *Gallicolumba criniger* |
| Philippine leafbird | *Chloropsis flavipennis* |
| Philippine eagle-owl | *Bubo philippensis* |
| Philippine kingfisher | *Ceyx melanurus* |
| Rufous lored kingfisher | *Todirhampus winchelli* |
| Bagobo babbler | *Leonardina woodi* |
| Red-eared parrotfinch | *Erythrura coloria* |
| Cryptic flycatcher | *Ficedula crypta* |
| Mindanao racquet-tail | *Prioniturus waterstradti* |
| Mindanao scops owl | *Otus mirus* |
| Philippine hawk eagle | *Spizaetus philippensis* |
| Lesser eagle owl | *Mimizuku gurneyi* |
| Silvery kingfisher | *Alcedo argentata* |
| Writhed hornbill | *Aceros leucocephalus* |
| Miniature tit-babbler | *Micromacronus leytensis* |
| Whiskered flowerpecker | *Dicaeum propium* |
| Celestial monarch | *Hypothymis coelestis* |

Mt. Apo (3,143 m high, tallest in the country; the park 72,113 ha) is an ASEAN Heritage Site and the most popular national park in Mindanao for both foreigners and students on vacation. The Department of Tourism organizes climbs to Mt. Apo usually in April-May and in October during the dry season. Park regulations have been set in place starting 1999. Not more than 2,200 climbers are allowed each year and only six entry points are used: Ilomavis in Kidapawan City, Magpet and Makilala in Cotabato, Kapatagan and Sibulan in Davao del Sur, and Calinan in Davao City. Climbers pay a registration fee, undergo briefing and orientation about the logistics and ethics of climbing and park use, and must bring complete mountaineering gear including tents and portable stoves. Prohibited are bringing big knives and axes, cutting trees or harvesting fruits and flowers on the mountain, washing with detergents and shampoo, and using pyrotechnics to celebrate the climb. Much habitat destruction and loss of biodiversity have been caused by tourism-related fires as well as drought-related wild fires during El Niño years.

From Kidapawan, climbers take a jeep to Ilomavis 700 m above sea level; from there, they trek or hitch rides with vehicles of the Philippine National Oil Company (PNOC). PNOC implements an active reforestation program on Mt. Apo. It also operates a visitors' center, Pook Kalikasan, with a nice botanical garden and a tribal livelihood training center, right next to the deep-blue, near-boiling Lake Agco at 1,200 m elevation. From Lake Agco to the Mt. Apo peak is a challenging trek through forests, meadows, hot and cold sulfur springs, waterfalls, and crater lakes. Experienced climbers make it to the peak in one day.

Scientific expeditions have found very high biodiversity on Mt. Apo. For example, Fr. Enrique Schoenig and other biologists from the University of San Carlos collected more than 390 species of plants along with 337 species of insects, including 40 species of mosquitoes (Schoenig et al. 1975). The Mt. Apo rain forest has 629 species (148 families) of ferns, mosses, and flowering plants including dipterocarps, almaciga, rattan, and orchids. Six plants are endemic: *Alseodaphne philippinensis*, *Cypholophus microphyllus*, *Nepenthes copelandii*, *Piperomia elmeri*, *P. apoanum*, and the Philippine oak *Lithocarpus* *submonticulus* (DENR-UNEP 1997). The giant flower rafflesia *Rafflesia schadenbergiana* was found for the first and last time on Mt. Apo in 1882 and is feared to be extinct (Heaney & Regalado 1998). The 'waling-waling' *Euanthe sanderiana* was first seen in 1882 and has been bred into many varieties with flowers as large as 7-12 cm.

Mt. Apo has 227 species of vertebrates in 69 families (DENR-UNEP 1997). Among the mammals are the Philippine deer, warty pig, civet, flying lemur, macaque, and several Mindanao-endemic insectivores, tree shrew, tree squirrel, bats, and rats. Among the rats on Mt. Apo are *Tarsomys apoensis*, *Limnomys sibuanus*, and *Apomys hylocetes* (Heaney et al. 1987). Lizards and skinks include *Otosaurus cumingi*, *Lipinia quadrivittata*. Frogs include *Rana magna*, *Leptobrachium hasselti*, and *Pelophryne brevipes*. Birds number 84 species, including the Philippine eagle, Philippine trogon, bullfinch, Bagobo babbler, Apo mynah, Apo lorikeet, and Apo sunbird. Among the 118 butterflies are five endemics: *Parantica schoenigi*, *Delias lecicki*, *D. schoenigi*, *D. apoensis*, *D. woodi* (DENR-UNEP 1997).

Mt. Kitanglad Natural Park in Bukidnon includes 29,716 ha of lowland to upper montane rain forests and many streams, a diverse vegetation (with 26 rare endemic plants), 17 mammals, many reptiles and amphibians, and 115 birds including the Philippine eagle, the Apo mynah, and the Apo sunbird (DENR-UNEP 1997). The Mindanao pygmy fruit bat *Alionycteris paucidentata* is known only from 2,250 m elevation in the mossy forests of Mt. Kitanglad (Heaney et al. 1997).

Mt. Malindang National Park (53,262 ha) in Misamis Occidental has a highly diverse flora and fauna including the Philippine tarsier, deer, macaque, warty pig, flying lemur, flying squirrel, palm civet, fruit bats, and rats like *Limnomys sibuanus*. The greater Mindanao shrew *Crocidura grandis* is known only from a single specimen taken in 1906 at 1,860 m on Mt. Malindang (Heaney et al. 1997). The diverse herpetofauna includes several new species of frogs.

Initao National Park (57 ha) between Cagayan de Oro and Iligan is a highly disturbed residual forest with the national highway cutting right through it. There still are dipterocarps, ferns, orchids, and some wildlife including bats. Caves and cliffs overlook beaches and a marine ecosystem with 64 species of corals, 85 seaweeds, 89 invertebrates, and many fishes (A. Galam, pers. comm.). Initao is a locally favored picnic area, but not well maintained.

Already a popular ecotourism destination, Camiguin Island (26,500 ha) has seven volcanoes, and several waterfalls, hot and cold springs, and a rich fauna including the unique Camiguin giant moss-mouse *Tarsomys sp.*, Mindanao shrew mouse, and the writhed hornbill (Heaney et al. 1997). Along the coast of Camiguin are found 106 species of seaweeds and seven species of seagrasses (O. Paderanga, pers. comm.). Camiguin should be managed properly for ecotourism before it is degraded by an increased influx of people.

The Siargao Island Wildlife Sanctuary (278,914 ha) in Surigao del Norte includes a vast mangrove forest (8,692 ha) and a pristine coral reef complex with 38 genera of corals, 59 seaweeds, 8 seagrasses, 137 mollusks, 106 fishes including the whale shark, 14 mammals including the dugong, many wetland birds, and lizards, marine turtles, and the saltwater crocodile (DENR-UNEP 1997). Very high biodiversity has been recorded in Siargao and Dinagat Islands, including 115 bird species, 58 amphibians and reptiles, various orchids and the Philippine ironwood *Xanthosternum verdugonianus* (DuPont & Rabor 1973, Ross & Lazell 1990, DENR-UNEP 1997). Among the mammals are the Dinagat hairy-tailed cloud rat *Crateromys australis*, the Dinagat gymnure *Podogymnura aureospinula*, and the shrew *Urogale everetti*, inhabitants of primary and secondary forests (Heaney et al. 1997). Bats on the islands include *Pteropus leucopterus* and *Eonycteris robusta*. Among the resident birds are the celestial monarch, writhed hornbill, lesser eagle owl, and the Mindanao bleeding-heart pigeon (Tabaranza & Mallari 1997).

Agusan Marsh (14,836 ha) and Liguasan Marsh (30,000 ha) are large, relatively undisturbed freshwater swamps that serve as catch basins and flood control systems in eastern and central Mindanao. Both are wintering grounds for hordes of migratory wading birds and home to the last large populations of crocodiles (Davies 1992). Agusan Marsh has 31 species of flowering plants and ferns, 10 mammals, many amphibians, 22 lizards, two crocodiles, 7 snakes, 102 birds including the Oriental darter, purple heron, and whistling duck, and 65 butterflies including the rare *Papilio antonio*, *Graphium cordus*, and *G. ideaoides* (DENR-UNEP 1997). Liguasan Marsh was declared a game refuge and bird sanctuary in 1941 and has stayed relatively pristine because of the unstable peace and order situation in the area. But there are now plans to drain Liguasan and convert it into the agricultural food basket of Mindanao, a development that would be disastrous for biodiversity and long-term economic growth.

Lanao Lake (180,460 ha, second largest in the country) was declared a watershed reserve only in 1992, but nearby Sacred Mountain and five other picturesque locations in Lanao del Sur (Lake Butig, Lake Dapao, Rungkunan, Salikata, Pantawaraya) were declared national parks in 1965 (DENR-UNEP 1997). Most of the mountains around Lanao Lake have suffered from heavy logging, but it is not known how much remains of the dipterocarp forests and the wildlife. Lanao Lake itself used to have a very diverse endemic fish fauna: 13 species of *Puntius* and five species in four new genera of *Cyprinidae* (Herre 1924, 1933). Of these 18 endemic carps, only the 'tumaginting' *Puntius sirang* can be fished at the present time; most of the others went extinct in the 1970-80s due competition and predation by introduced fishes such as the goby *Glossogobius giurus* (Escudero et al. 1980). Unfortunately, biodiversity studies are difficult to conduct in the lakes and wilderness of Lanao.

Lanao Lake is the lifeblood of about a million Muslim Maranaos dependent on it and the surrounding watershed for water, fish, rice, livelihood, transportation, and cultural and religious identity. Unstable political relations with the Christians have been aggravated by the use of Lanao Lake and Agus River for megahydropower. Maria Cristina Falls downstream can now be seen only by appointment and only in half its glory because of the hydropower operations, but the area around it is well preserved.

Lake Sebu is much smaller, only 374 ha in surface area, but has several adjacent lakelets that attract tourists into T'boli highlands. Unfortunately, Lake Sebu has become polluted and prone to fish kills due to intensive tilapia farming (Z. Beniga, pers. comm.). Nearby Lake Buluan (6,300 ha) was declared a game refuge and bird sanctuary as early as 1926, but is now crowded with tilapia cages and milkfish pens.

The Greater Sulu faunal region is home to the Philippine slow loris *Nycticebus coucang menagensis*, the rare gray flying fox *Pteropus speciosus*, and the endemic Tawitawi Island shrew rat *Rattus tawitawiensis* (Fooden 1990, Heaney et al. 1997). Also found here are about 53 species of birds, including the endangered Sulu bleeding-heart dove *Gallicolumba menagei*, the blue-winged racquet-tail *Prioniturus verticalis*, and the Sulu hornbill *Anthracoceros montani* (Tabaranza & Mallari 1997). Dugongs may still have viable populations along the southern Mindanao coast to Tawitawi (WCSP 1997).

The Turtle Islands Wildlife Sanctuary includes 392 ha of islands and 136,000 ha of reefs. The islands harbor 80% of the marine turtles in the Philippines and are the only green turtle rookery in the ASEAN region (WCSP 1997). The sanctuary also has 40 genera of corals, 361 species of fish, 65 seaweeds, four seagrasses, 12 birds, lizards, and bats (DENR-UNEP 1997). There are mud volcanoes in Lihiman and Langaan Islands. The declaration of the transborder Turtle Islands Heritage Protected Area is expected to ensure the protection, management, and conservation of marine turtles through cooperation with Malaysia.

## Ecotourism and Environment Education

Protected areas are important to set up and maintain for several purposes such as: (i) *in situ* conservation of flora and fauna in their native and large enough habitats, (ii) as storehouse of biological and genetic information, (iii) continuance of ecosystem functions, (iv) production of extra biomass for harvest on sustainable basis, (v) education. Establishment and management of protected areas are often difficult, expensive, and subject to conflicting considerations, both in developing and developed countries. It is now generally agreed that for protected areas to be viable in the long run, they must be part of integrated resource management, and they must permit some degree of sustainable use by the local population (White & Palaganas 1991, Davies 1992, Kenchington & Bleakley 1994, DENR-UNEP 1997).

Regulated ecotourism for environment education is one such sustainable use and one that generates income and responsible citizens. Ecotourism involves learning about the forests, caves, swamps, reefs, lakes, and volcanoes in the country. It involves leaving the comforts of modern living to immerse in natural habitats, encounter wildlife, feast the eyes on the beauty of creation, heal spiritually, and finally to act responsibly. Ecotourism can be integrated with school, family, and business activities, and tourist establishments become more attractive when they incorporate, or convert to, ecotourism. Accessibility and enjoyment of the protected areas may be improved by providing well-marked trails with ground cover, well-trained park wardens, and well-researched information materials. Travel is an effective educator and should be made more affordable and popular, along the lines of "Magbalik-probinsiya!" of the Department of Tourism.

More research has to be done on Philippine flora and fauna to produce scientific reports and popular materials for scientists, teachers, tourists, and children. Studies on the taxonomy and biology of plants and animals are required for their conservation and management. But taxonomy is a much neglected discipline in the Philippines and species lists are difficult to put together and those of invertebrates in particular are likely to be erroneous. The country has 13,500 species of plants (5% of the world's flora, with 3,500 endemic species and 25 endemic genera), including 800 species of orchids, 950 ferns, and nearly 1,000 green, red, and brown algae (DENR-UNEP 1997). The invertebrate fauna includes about 20,000 species of insects, 8,000 mollusks, 2,000 crustaceans, and 500 corals. About 2,175 species of fish and 1,150 species of non-fish vertebrates live here — 95 amphibians, 266 reptiles, 558 birds, 180 native land mammals, and 20-25 marine mammals (DENR-UNEP 1997, WCSP 1997). The herpetofauna and mammalian fauna are quite well known (Alcala 1986, Rabor 1986, Heaney et al. 1987, 1997), and the birds even more so, having been described in several illustrated books (e.g., DuPont 1971, Dickinson et al. 1991), which however are not widely available.

Information about the protected areas and biodiversity (ecological value, threats, etc.) must be widely disseminated, as part of the science and geography lessons as early as primary school, and through various effective media (colorful and informative leaflets, brochures, books, videos) for the general public. More writers and artists are needed to explain biodiversity, conservation, and other environment issues in simple language. Recently, the beauty and wonder of Philippine biodiversity have been captured on film and in several books, especially by Bookmark (Springsteen & Leobrera 1986, Chou & Ali´ o 1992, Tan 1992, 1994, Madulid 1995, WCSP 1997, Yin 1997, DENR-UNEP 1997, Fernandez 1997, Aguilar & Bortles, 1997, Alcala & Brown 1998 Heaney & Regalado 1998). Elementary schools, high schools, and homes should build up a Filipiniana library including these and other recent books about the national heritage (e.g., Best et al. 1996, Paterno et al. 1996, Magdaraog 1998). When environment education and biodiversity conservation succeed, the Philippines may have a chance at real progress.

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RESEARCH

# Metabolism of a Northwestern Philippine Coral Reef Flat Measured at Two Spatial Scales

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**In order to determine whether the scale of measurement of primary production and respiration in a coral reef has an effect on the result, a direct comparison of open flow and enclosure techniques was carried out in a tropical coral reef flat. The spatial scales concerned were hundreds of square meters versus square centimeters, respectively. Using open flow methods, the reef flat was found to have a net positive primary production during the day, and respiration at night, conforming to what has been established for these systems. Metabolic rates measured with both techniques were closely similar, indicating that artifacts possibly introduced by the enclosure method were negligible. With the enclosure technique, however, it was possible to detect discrete differences among substrates (coral, rubble and sand), as well as specific responses to environmental factors such as light, temperature and salinity. On the other hand, measurements using open flow techniques yielded a greater variability in rates so that significant effects of environmental factors were not evident on the larger scale. However, differences in reef metabolism between the dry-cool and wet seasons which the enclosure method failed to detect were shown to be significant using results derived from fixed station respirometry. It is clear that measurements at the larger scale (open system) encompass more factors and processes than do those at the smaller scale (enclosure). Results obtained using the latter, however, are more precise and replicable, and help to explain the more complex patterns that emerge at the broader system level.**

**Key words:** *System metabolism, enclosure method, primary production, open flow respirometry*

Coral reef ecosystems are recognized for their relatively high gross primary productivity and the diversity of life they support. Direct measurements of reef productivity are known from work commencing in the middle of the century (Odum & Odum 1955, Kinsey 1985a). It has also been established that coral reefs are divided into zones which have different levels of gross primary productivity, and varying production:respiration (P/R) ratios (summarized in Kinsey 1985a, Wiebe 1988). Thus, some zones which are composed predominantly of live coral are constituted as autotrophic systems which are presumed to export organic material to zones which are heterotrophic, such as sand communities.

One method in the study of reef productivity involves the use of *in situ* enclosures to isolate specific components such as single organisms or patches of substrate, and to measure their metabolism (Zeitzschel 1981, Hopkinson et al. 1991, Nacorda & Yap 1996). Such an approach renders an advantage in the ability to replicate the experimental unit (in this case, a particular ecological component) to derive an acceptable amount of statistical rigor. The technique also enables relative control of environmental conditions within the enclosure, so that factors of interest are segregated from what might be considered "noise" in the larger system (e.g., nuisance organisms, sporadic turbulence). The enclosure technique, for example, made it possible to detect significant correlations between metabolism (both at the organism and at the community level) and environmental parameters such as light (subsurface irradiance), temperature and salinity (Yap et al. 1994).

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A disadvantage of the method lies in the possibility that enclosed conditions promote the abnormal proliferation of microorganisms which may distort metabolic profiles (W. Wiebe, pers. comm.). Variations in water movement within the enclosure would also cause significant changes in production and respiration of the organism or community being incubated (Lesser et al. 1994, Montebon & Yap 1995). Another argument against the approach is that individual measurements on reef components cannot be added up to yield a single measure that is representative of the larger system (Mann 1988).

A whole suite of techniques has been developed that circumvents the above limitations by directing measurements at parameters that are believed to integrate metabolism at the system level, such as dissolved oxygen and carbon dioxide dynamics (Kinsey 1978, Smith 1981, Barnes & Devereux 1984, Kinsey 1985a,b). In this way, the P/R ratios of entire reef zones have been estimated, supporting the notion that there are varying degrees of autotrophy and heterotrophy within a reef system. These procedures are referred to as "open flow" (as opposed to "enclosure") methods (Kinsey 1985b).

The present paper arises from work involving the measurement of primary production and respiration of ecological components of a coral reef flat at two scales: on the order of square centimeters with an associated water volume of several liters (the enclosure technique); and tens to hundreds of square meters involving volumes of hundreds to thousands of liters (the open flow method). The specific objective of this study is to compare the open flow and enclosure techniques in terms of measuring the primary production and energy consumption of a coral reef flat, including seasonal patterns.

A central theme in ecology is that of scale (Hatcher 1990, Levin 1992). The present investigation explores the question of whether measurements at different scales yield different "pictures" of the same system, or, viewed in a different way, if different aspects of the same processes are captured.

Open flow measurements of the coral reef flat of interest in this paper were made using variations of the techniques refined by Kinsey (1985b). These made use of a single fixed station, two fixed stations, and a moving station. Results of these measurements are compared with those from incubations of individual coralla or patches of substrate done within the same period of time in the same area, as well as with results from a 2-yr study using the same enclosure technique (Yap et al. 1994).

## Materials and Methods

All measurements were conducted on a reef flat in the northern Philippines at approximately 16°24'41"N and 119°54'25"E (Yap et al. 1994). The reef flat is 0.5 km wide and has an average water depth of 1 m. A sea grass zone extends from the shore about halfway across the flat, then gives way to a sand-rubble substrate associated with coral, predominantly large microatolls of the genera *Porites* and *Montipora*. Other conspicuous corals belong to the Fungiidae and the Faviidae.

A year in the Philippines is roughly characterized into a dry-cool, a dry-warm, and a wet season (Yap et al. 1994). The reef flat was visited for several consecutive days (in order to obtain independent replicate measurements) in April 1992, Sep 1992, May 1993 and Oct 1993. April and May represented the dry-warm period, while September and October were wet months.

### Open flow respirometry

On each occasion, a light boat containing the requisite instrumentation was moored at approximately 1 m depth in the coral-rubble-sand zone and constituted the fixed station for open flow respirometry (Kinsey 1985b). Measurements over a 24-h cycle were carried out on 27-28 April 1992. During other visits, fixed-station measurements were usually done between 0900-1600H, with 2 replicates 200-300 m apart in the same general area.

When water flowed in a consistent direction between the 2 fixed stations for at least several hours (usually 3), measurements were recalculated so that the metabolic activity of the benthic community between the 2 stations was determined (2-station open flow respirometry, Kinsey 1985b). Replication was done by repeating the measurements over 2 or 3 d during which the same meteorologic conditions prevailed.

To perform moving station respirometry (Kinsey 1985b), a water mass was marked with fluorescein dye and a subsurface drogue which were followed with a light boat while a dissolved oxygen probe was kept immersed in it. The subsurface drogue was a plastic bottle filled with water, allowing enough air to be trapped at the neck to keep the bottle upright. It was submerged except for the top so that it was not dragged along by the wind. The design was recommended by Dr. Donald Kinsey (pers. comm.) as being among the most reliable and satisfactory.

The water mass travelled over a variety of zones on the reef flat, allowing their metabolic activity to be determined. The general type of bottom cover and dominant organisms were visually estimated. Replication again was achieved through repetition of the measurements over 2-3 d under the same meteorologic conditions. Moving station measurements during daylight hours were performed on 2 and 4 May 1993 and 17-18 October 1993 ( see Table 1).

In addition, 24-h measurements of the same reef flat using moving station respirometry were carried out on 12-13 April, 14-15 April and 18-19 April 1994, yielding 3 independent replicate samples.

In all of the above exercises (with the exception of the 24-h runs), photosynthetic and respiration rates were computed only for the times water was flowing in a consistent direction for at least 0.5 h. This length of time was considered sufficient for significant metabolic activity of the benthos to be detected. Current velocity was measured by attaching a string to the drogue and measuring the length paid out after 1 minute. Direction was read with a hand-held compass. This procedure was repeated at least 3 times during each 0.5 h run.

Dissolved oxygen was recorded continually at the bottom and at the surface (to later compute for % saturation) using a dissolved oxygen probe (Nester Instruments model 8500x) which was routinely calibrated

**Table 1.** Numbers of replicate samples used for each technique over the duration of the experiment. A "replicate" is a measurement taken during which water flow was unidirectional for at least 0.5 hr. This table excludes the 24 hr measurements, 1 of which was conducted using the single station technique, and 3 using the moving station technique (see text for details).

| | Single Station | Two Stations | Moving Station | Alkalinity Anomaly |
|---|---|---|---|---|
| 1992 | | | | |
| DRY | Day 1: n=6 | n=2 | | |
| | Day 2: n=3 | | | AM: n=3 |
| | | | | NN: n=3 |
| | | | | PM: n=6 |
| WET | Day 1: n=4 | n=4 | | |
| | Day 2: n=3 | | | AM: n=3 |
| | | | | NN: n=3 |
| | | | | PM: N=6 |
| 1993 | | | | |
| DRY | Day 1: n=2 | | n=9 | |
| | Day 1: n=2 | | | AM: n=3 |
| | | | | NN: n=3 |
| | | | | PM: n=6 |
| | Day 3: n=2 | | n=4 | |
| WET | Day 1: n=3 | | n=8 | |
| | Day 2: n=3 | | n=4 | |
| | Day 3: n=3 | | | AM: n=3 |
| | | | | NN: n=3 |
| | | | | PM: n=6 |

in air during each field visit following manufacturer's instructions. The probe had a resolution of 0.01 mg $L^{-1}$. Other parameters measured were water temperature, depth, salinity, wind velocity (estimated using the published Beaufort scale), and subsurface irradiance in microEinsteins $m^{-2}$ $s^{-1}$ (using a LI-COR 193SA spherical quantum sensor).

Data were processed according to formulas developed and published by Kinsey (1985b). Briefly, hourly rate of net photosynthesis (y) or hourly rate of respiration (r) was computed as follows:

$$y \text{ (or } r) = dM/dt + DK \qquad (1)$$

where $dM/dt = (dC/dt)Z$

and $K$ = areal diffusion coefficient for $O_2$

$C$ = concentration of $O_2$

$t$ = time interval

$Z$ = water depth

$D$ = mean value of saturation excess or deficit at the water surface during the period used for experimental determination of $dC/dt$

D was computed from actual values of dissolved oxygen concentrations at the sea surface which were converted to saturation values using actual temperature and salinity measured at the time of the experiment. K was computed using the formula:

$$K = 4.45 \times 10^{-3} V^{2.095} + 0.50 \qquad (2)$$

where V is wind speed in knots. The assumption here is that diffusion of oxygen is proportional to wind speed.

To ensure the precision of the technique, care was taken to confine all measurements within the limits set by Kinsey (1985b), viz., water depths of approximately 1 m or less, and residence times of the water of at least 0.5 h.

### Comparison of open flow respirometry with the enclosure method

To compare the open flow techniques above with the enclosure method, incubations of individual coralla of the family *Fungiidae*, and of patches of rubble and sand from the same area were made during the same periods above. Methods briefly described below are detailed in Yap et al. (1994).

Members of the family Fungiidae were used to represent the coral component (Yap et al 1994). A different coral specimen each time was incubated in a watertight acrylic chamber (volume approx. 9 L). Metabolic measurements were standardized for the size of the individual corallum (measured as projected area) and then extrapolated to unit area ($m^2$).

For the rubble and sand, a patch of substrate was enclosed by a metal cylinder (diameter approx. 20 cm, height approx. 10 cm) over which the acrylic cylinder was fitted. The top end of the cylinder contained the dissolved oxygen probe and a battery operated bilge pump to stir the enclosed water.

Two replicate runs for both photosynthesis and respiration were made for each component during each visit to compare with results from open system measurements. The data were additional to those collected over a 2-yr observation period of the same reef components utilizing the same methods (Yap et al. 1994). The enclosure measurements were thus replicated monthly over a time span of at least 2 years. The results obtained so far demonstrate the robustness of this method.

### Statistical analyses

Differences in oxygen evolution or consumption rates between seasons were tested for using the analysis of variance (ANOVA). Assumptions of normality and homoscedasticity were met by transforming the data (Sokal & Rohlf 1981, Zar 1984). Possible effects of environmental parameters on net primary production were examined using multiple linear regression analysis (Sokal & Rohlf 1981, Zar 1984).

## Results

### 24-h fixed station respirometry

Metabolic measurements of the coral-rubble-sand zone from the fixed station over 24 h displayed the expected trend of over-all positive net primary production during the day (0700-1800H), and respiration or energy consumption after light values had dropped below the compensation point (about 1800H) (Figure 1). Net photosynthesis ranged between 0.084-3.012 g $O_2$ $m^{-2}$ $h^{-1}$, with maximum levels attained between 1430-1500H. Nighttime respiration was measured at 0.129-2.811 g $O_2$ $m^{-2}$ $h^{-1}$, with highest values between 1930 and 2000H, or

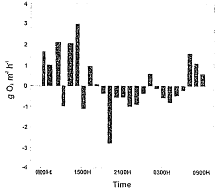

**Figure 1.** Net photosynthesis and respiration of a coral-rubble-sand zone measured over a 24-h period (27-28 Apr 1992) from a fixed station using open flow respirometry techniques. Dotted line is a fifth order regression line generated using SigmaPlot v4.1 (Jandel) to indicate approximate trend.

shortly after nightfall. Digitizing the curve (Figure 1) yielded a value for daytime net photosynthesis of 9.480 ± 0.065 g $O_2$ $m^{-2}$ $d^{-1}$, and nighttime respiration of 6.489 ± 0.043 g $O_2$ $m^{-2}$ $d^{-1}$. Community P/R (net primary production:nighttime respiration) was 1.46, so that the reef flat community was generally autotrophic over a 24-h period.

The net photosynthesis during the day is clearly a function of light (see Figure 2). Negative net photosynthetic rates were measured during the day, particularly at 1230-1300H with a value of -1.005 g $O_2$ $m^{-2}$ $h^{-1}$, and at 1530-1600H with a value of -1.103 g $O_2$ $m^{-2}$ $h^{-1}$. Similarly, there was a single anomalous reading of +0.592 g $O_2$ $m^{-2}$ $h^{-1}$ at 0130-0200H (Figure 1). Measurements of the wind field during that same 24-h period indicated changes in wind direction (see Figure 3) which altered current direction in the reef flat. Because of the shallow depths involved, the current was observed to be predominantly wind driven. Current shifts could have brought in water masses from surrounding areas which were relatively depleted or enriched in dissolved oxygen, thus explaining the anomalous readings obtained during the day or night.

### Metabolism of different zones of the reef flat measured using moving station respirometry

The types of zones observed were as follows: pure sea grass stands, mixtures of sea grass and

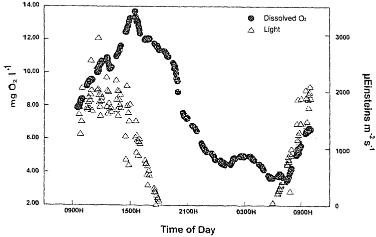

Figure 2. Dissolved oxygen and subsurface irradiance measured at the coral-rubble-sand zone over a 24-h

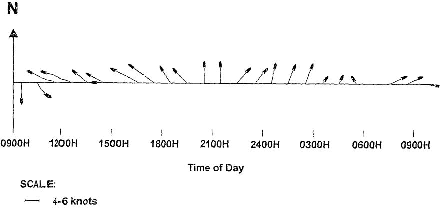

Figure 3. Wind velocity (converted from the Beaufort scale) measured at the coral-rubble-sand zone over a 24-h period (27-28 April 1992).

rubble, sea grass and algae, sea grass and coral, pure algal stands (mostly *Sargassum*), mixtures of algae and rubble, algae and coral, and mixtures of coral and rubble. The highest net primary production rates at times of peak light intensity were found for the pure algal stands and the coral-algal communities during the dry season. Rates were 5.05 and 5.47 g $O_2$ $m^{-2}$ $h^{-1}$, respectively (see Figure 4). A greater number of zones were measured during the dry season sampling (May 1993) than during the wet season (October 1993) due to the path taken by the water mass being monitored.

Twenty-four hour measurements of the same reef flat using moving station respirometry depicted the trend described in the preceding section of over-all net positive primary production during the day and of respiration or oxygen consumption during the night ( see Figure 5 a, b, c). Observations were made over 3 successive 24-h periods during the summer in April 1994. All 3 replicate runs, however, included an observation of net oxygen consumption during the day some time between 1100H and 1500H. Measured rates in these instances ranged from –0.0299 to –24.487 g $O_2$ $m^{-2}$ $h^{-1}$. The negative values could have been caused by a predominance of respiration by heterotrophs over photosynthesis by primary producers in particular zones.

The results here are similar to those obtained using

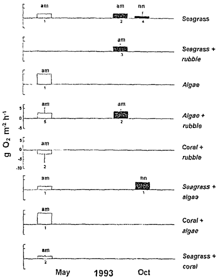

Figure 4. Net primary production of different zones on the reef flat measured in May (dry season) and Oct (wet season) 1993 using moving station respirometry. Error bars are standard deviations. Numbers below bars indicate replicate measurements. Open and filled bars indicate dry and wet seasons, respectively.

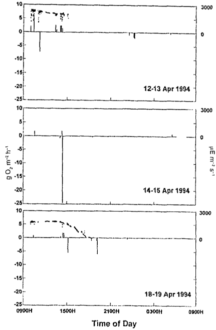

**Figure 5.** Net primary production and respiration of the reef flat measured over 24-h using moving station respirometry over 3 replicate periods. a) 12-13 Apr 1994; b) 14-15 Apr 1994; c) 18-19 Apr 1994. Bars indicate net primary production or respiration, and points are corresponding light values for the times indicated.

the fixed station method (see preceding section). Net positive primary production values ranged between 0.05 and 5.816 g $O_2$ $m^{-2}$ $h^{-1}$. Hours with peak values were not consistent among the replicate runs. Nighttime respiration rates were measured at 0.005-6.044 g $O_2$ $m^{-2}$ $h^{-1}$.

## Comparison of enclosure and open flow measurements

Net photosynthetic rates of a coral-rubble-sand zone using single fixed station respirometry, when compared with measurements of the same reef components incubated within enclosures, namely, single coralla (of the family Fungiidae), rubble and sand, were found to be within the same order of magnitude, specifically, within 5 g $O_2$ $m^{-2}$ $h^{-1}$ (Figure 6).

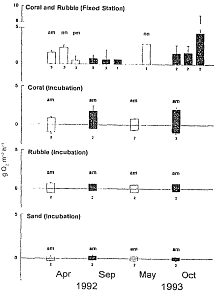

**Figure 6.** Comparison of fixed station measurements of net primary production of a coral-rubble-sand zone using open flow respirometry with results of incubations of coral, rubble and sand using *in situ* enclosures. Values for the dry-warm season and the wet season are indicated by open and filled bars, respectively. Negative values from the substrate incubations denote respiration. Error bars are standard deviations. Numbers below bars indicate replicate measurements.

There were differences in both primary production and respiration among coral, rubble and sand (Figure 6) which were statistically significant in the previous study (Yap et al. 1994). The latter results showed an absence of seasonal differences (e.g., between the dry-warm and wet periods) in the metabolism of the *incubated* substrates which is also supported by additional observations in this study (Figure 6). Yap et al., however, determined significant correlations between gross photosynthesis and respiration and environmental parameters such as light, temperature and salinity (see Table 2) at the scale of the field enclosure.

Mean values for light, water temperature, salinity, current speed, wind force and depth for April 1992 and May 1993 (representing the dry-warm season) and for September 1992 and October 1993 (representing the wet season) are characterized by variability, with significant differences between seasons seen only for salinity (see Table 3). A multiple regression test did not elicit any significant associations of system primary production and respiration with any of the environmental factors measured ($p>0.05$).

An ANOVA revealed significant differences between seasons of net primary production rates of the coral-rubble-sand zone measured using single fixed station respirometry (see Table 4). With the exception of an obvious outlier among the afternoon values for October 1993, net system primary production in the dry-warm season was seen to be higher than during the wet (Figure 6). There were no significant differences between years, and the year x season interaction term was insignificant.

**Table 2.** Results of regression analysis of gross primary production and respiration of coral, rubble and sand against light, temperature and salinity. Only significant results are given. Data are from October 1989 to November 1991. * $p<0.05$. (From Yap et al. 1994).

| | Environmental factor | Coefficient | $R^2$ |
|---|---|---|---|
| Coral | | | |
| Production | Light | 0.588* | 0.250 |
| Respiration | Temperature | 51.312* | 0.238 |
| Rubble | | | |
| Production | Light | 0.234* | 0.250 |
| | Salinity | -74.190* | |
| Respiration[a] | Salinity | -0.044* | 0.058 |
| Sand | | | |
| Production | Light | 0.092* | 0.178 |
| | Salinity | -34.084* | |
| Respiration | | | |

[a] Values log-transformed

The detection of a seasonal difference in system metabolism with single fixed station respirometry was repeated in one instance using 2 fixed stations employing the same open flow technique (see Figure 7). Measurements during the dry season (April 1992) showed net positive primary production of the reef flat, while observations during the wet season (September 1992) yielded net consumption or respiration during the same daytime hours (1000-1200H).

## Discussion

Hourly measurements of net primary production and respiration on the same reef flat using either the enclosure or the various open flow techniques yielded similar values. These findings are reinforced by those of another study which also attempted a direct comparison of enclosure and open flow techniques — that of Murphy & Kremer (1983) in a coral atoll in the eastern Pacific. They found good agreement between total system and separate component estimates of metabolism. Thus, whatever artifacts which were inherent in the enclosure

**Table 3.** Mean values (± S.D) of subsurface irradiance, water temperature, salinity, current speed, wind force and depth from four sampling periods (April and September 1992; May and October 1993) representing two seasons (dry-warm and wet). Values in parentheses indicate sample sizes.

| | Light ($\mu E\, m^{-2} s^{-1}$) | Water (°C) | Salinity (‰) | Current Speed ($m\ min^{-1}$) | Wind Force (Beaufort Scale) | Depth (m) |
|---|---|---|---|---|---|---|
| April 1992 (DRY) | 1472 ± 416 | 31.8 ± 0.8 | | 1.83 ± 0.60 | 7 ± 1 | 1.42 ± 0.23 |
| | (9) | (9) | 34 | (5) | (6) | (9) |
| Sept 1992(WET) | 1339 ± 333 | 29.8 ± 0.2 | | 7.91 ± 6.30 | 9 ± 3 | 1.23 ± 0.30 |
| | (5) | (7) | 28 | (7) | (5) | (7) |
| May 1993(DRY) | 1742 ± 271 | 31.3 ± 0.8 | | 3.40 ± 0.97 | 5 ± 2 | 1.05 ± 0.08 |
| | (6) | (6) | 34 | (4) | (6) | (6) |
| Oct 1993(WET) | 1566 ± 407 | 31.4 ± 1.2 | | 4.07 ± 1.9 | 5 ± 1 | 0.95 ± 0.12 |
| | (9) | (9) | 30-32 | (9) | (9) | (9) |

**Table 4.** Results of two-way ANOVA testing for significant differences in system net primary production measured using single fixed station respirometry between seasons (dry-warm and wet) and years (1992 and 1993). Data from different times of day were pooled, as no significant differences among times of day were detected.

| Sources of Variation | Sum of Squares | DF | Mean Square | F | Sig of F |
|---|---|---|---|---|---|
| Main Effects | 5.535 | 2 | 2.768 | 4.227 | 0.026 |
| Year | 2.712 | 1 | 2.712 | 4.142 | 0.052 |
| Season | 3.559 | 1 | 3.559 | 5.437 | 0.028 |
| Two-Way Interactions | 0.040 | 1 | 0.040 | 0.062 | 0.806 |
| Year | 0.040 | 1 | 0.040 | 0.062 | 0.806 |
| Explained | 5.654 | 3 | 1.885 | 2.879 | 0.055 |
| Residual | 17.023 | 26 | 0.655 | | |
| Total | 22.677 | 29 | 0.782 | | |

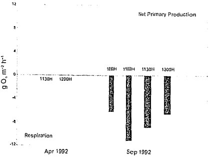

**Figure 7.** Metabolic activity (net primary production or respiration) of the reef flat measured from 2 fixed stations using open flow respirometry during the dry (Apr) and wet (Sept) season (1992). Each value represents measurements over one half-hour period.

method produced effects that were small and relatively insignificant compared to the metabolic rates measured.

Both fixed and moving station open flow techniques employed over a 24-h period in the reef flat showed results of over-all net positive primary production during the day, and energy consumption at night. All values were within range of those reported in previous studies (reviewed below). Coral reef flats are considered to have a slight excess of organic production probably due to the activity of short-lived filamentous algae (Barnes et al. 1986). The maximum respiration values observed shortly after nightfall at the fixed station could be due to the availability of significant amounts of substrates from photosynthesis generated during the day, and which are diminished in the course of the night (Porter et al. 1984).

"Anomalous" readings obtained with the open flow technique showed incidences of apparent respiration during the day, and apparent net production at night. This could be explained by the occurrence of water masses with either higher or lower dissolved oxygen content than the surrounding water body, with which the dissolved oxygen probe happened to come in contact. Such discrepancies, however, occurred only during the 24-h measurement periods, when shifts in water current were unavoidable. Anomalous readings were not obtained during the discrete 0.5-h measurements which were conducted only during times of unidirectional water flow. Since results from use of both open flow and enclosure techniques were in good agreement (Figure 6), then effects of atmospheric diffusion could be considered insignificant compared with effects due to bottom photosynthesis or respiration.

Furthermore, significant respiration due to possible degradation of seaweed or seagrass detritus in the water column could be ruled out. At the beginning of the study, control runs of water column metabolism using enclosures were carried out, and this was found to be undetectable. In terms of total reef activity, the water column is considered to be an almost negligible component (Kinsey 1978).

Data from the application of certain open flow techniques (single fixed station, 2 fixed stations) suggest seasonal differences in reef flat metabolism. The dry season measurements showed greater net positive primary production, while the wet season observations were of a mixture of energy production and consumption, even during the same daylight hours. A possible reason for the wet season trends is that most days are overcast, so that the limited available light reaching the photosynthetic organisms cannot sustain enough primary production to support the energy needs of the entire community over prolonged periods. Actual observations of *in situ* photosynthesis and respiration of rubble substrates have shown that, at a light level of about 400 µEinsteins m$^{-2}$ sec$^{-1}$, metabolic activity switches from net positive primary production to respiration (unpublished data). Light intensities during typical cloudy and rainy days have been observed to be close to this value for the water depths used in this study. In a higher latitude coral reef at 29° S, Crossland (1984) established a reduction in irradiance from summer to winter by 65%, thereby contributing to reduced net primary production of *Acropora formosa*. It is to be noted that, in the present study, *net* primary production is related to actual "yield" of a system (Lindeman 1942).

Values for the metabolism of various reef components determined in this study are seen to lie on the upper end of the range reported in the literature. They may be considered to be representative of the performance of *tropical* systems which are known to have relatively high rates of energy production and consumption.

Values published in the literature on the metabolism of various coral reef components span two orders of magnitude. For coral sand at 5-15 m depth off central Vietnam, Sorokin (1991) measured primary production rates of 0 - 0.75 g C m$^{-2}$ d$^{-1}$. These translate roughly to 0 - 0.25 g $O_2$ m$^{-2}$ h$^{-1}$. Sorokin (1990) reports a range of 5-20 g C m$^{-2}$ d$^{-1}$ and Pichon (1997) reports a similar range of 2.6-19.6 g C m$^{-2}$ d$^{-1}$ for various reefs in the Indo-Pacific (approximately 0.87-6.67 g $O_2$ m$^{-2}$ h$^{-1}$).

For purposes of interconverting values of carbon and oxygen, metabolic quotients are assumed to be equal to 1.0 (Kinsey 1978) for all autotrophic coral reef components. There is at least one known attempt to derive metabolic quotients for scleractinian corals. This is the work of Gattuso & Jaubert (1990) on *Stylophora* in the Red Sea who derived a PQ of 1.14-1.57, and an RQ of 0.65-0.99. These values, however, varied with depth.

Examples of recent work reporting values at the lower end of the range are mostly laboratory investigations on scleractinian corals. Kühl et al. (1995), working with *Favia* and *Acropora* from the Gulf of Aqaba, measured gross photosynthesis to be 0.54 g $O_2$ m$^{-2}$ h$^{-1}$, and respiration to range from 0.06 (dark) to 0.42 (light) g $O_2$ m$^{-2}$ h$^{-1}$. Lesser et al. (1994) determined a net photosynthetic rate of 0.64-1.6 g $O_2$ m$^{-2}$ h$^{-1}$ and respiration of 0.32-1.28 g $O_2$ m$^{-2}$ h$^{-1}$ in *Pocillopora damicornis* from Hawaii. Patterson et al. (1991) measured net photosynthesis up to 0.46 g $O_2$ m$^{-2}$ h$^{-1}$ and respiration up to 0.30 g $O_2$ m$^{-2}$ h$^{-1}$ in *Montastrea annularis* from St. Croix. In the Philippines, Montebon & Yap (1995) obtained values for net photosynthesis up to 0.29 g $O_2$ m$^{-2}$ h$^{-1}$ and respiration up to 0.21 g $O_2$ m$^{-2}$ h$^{-1}$ for branch tips of *Porites cylindrica* collected from the same reef flat as the present study.

Excellent agreement between results obtained from measurements in the laboratory and the field using closed systems but different dissolved oxygen techniques was achieved for various specimens of *Fungia* which were gathered from the same shallow reef flat (Yap et al. 1994, 1995). As measured in both the laboratory and the field, theoretical maximum gross photosynthesis was 2.5 g $O_2$ m$^{-2}$ h$^{-1}$. Respiration in the laboratory was determined to be 0.5 g $O_2$ m$^{-2}$ h$^{-1}$, while in the field it was 0.8 g $O_2$ m$^{-2}$ h$^{-1}$

Values approaching the higher end of the range reported in the literature were obtained mostly by investigators using *in situ* enclosures (i.e., in the field) to measure metabolic rates of various reef components. Tun et al. (1994a) experimented with the hard corals *Fungia*, *Goniopora*, *Heliofungia* and *Platygyra* in Singapore, and found maximum gross photosynthesis to range between 1.056 and 4.416 g $O_2$ m$^{-2}$ h$^{-1}$, and respiration to range between 0.256 and 0.96 g $O_2$ m$^{-2}$ h$^{-1}$. Rogers & Salesky (1981), working in St. Croix, determined net photosynthesis and respiration of macroalgae to be 1.16-8.98 and 0.19-0.64 g $O_2$ $m^{-2}$ $h^{-1}$, respectively. For *Sargassum baccularia* in the Central Great Barrier Reef, Schaffelke & Klumpp (1997) measured net photosynthetic rates of 0.13-1.3 g $O_2$ $m^{-2}$ $h^{-1}$ (0.4-3.0 g C $m^{-2}$ $d^{-1}$). For *Enhalus* (a seagrass) and *Sargassum* in Singapore, maximum gross photosynthetic rates were 9.09 and 15.47 g $O_2$ $m^{-2}$ $h^{-1}$, respectively, while respiration values were 0.82 and 1.1 g $O_2$ $m^{-2}$ $h^{-1}$, respectively (Tun et al. 1994b). All these results agree well with those of the present study.

In his review of system metabolic values for coral reefs, Kinsey (1985a) includes figures of 17-37 g C $m^{-2}$ $d^{-1}$ at the higher end of the reported range for gross primary production in various localities. These convert to rates of 5.7-12.3 g $O_2$ $m^{-2}$ $h^{-1}$, assuming an 8-hour period for photosynthesis when sufficient light penetrates the water column to evoke a photosynthetic response in the benthos (due to relatively high compensation light intensities — see discussion below). In terms of respiration, Kinsey (1985a) quotes high values of 17-24 g C $m^{-2}$ $d^{-1}$. These translate to 1.89-2.67 g $O_2$ $m^{-2}$ $h^{-1}$, assuming a full 24-h period for respiration. A comparison of these respiration figures with the gross photosynthetic measurements covered in Kinsey's review implies relatively high rates of net primary production.

On the upper extreme, Adey (1983) reports diel gross photosynthesis of up to 80 g $O_2$ $m^{-2}$ $d^{-1}$, and respiration of up to 2.6 g $O_2$ $m^{-2}$ $d^{-1}$ for a reef in St. Croix. Adey's measure of diel gross photosynthesis would be way above the value obtained in this study.

## Conclusion

The results of this study indicate that it is possible to study a natural ecosystem using two approaches: by conceptually breaking the system down into its ecological components and measuring their individual performance (the reductionist point of view, Pomeroy et al. 1988), or to target some integrated measure of whole system activity (the open flow techniques). Each approach yields valid data that should be treated as complementary.

When components are isolated, it is possible to measure differences among them, as well as responses to specific forcing factors such as physicochemical parameters (Yap et al. 1994). However, this approach failed to detect seasonality in metabolism of the bigger system, whereas the open flow techniques seemed to approach this capability. This implies that the latter encompass a larger scale (Hatcher 1990, Levin 1992), where more processes come into play than are evident at the level of the enclosure. It is also important to note that, for this reason, rates measured at the scale of the broader system possess more inherent variability (e.g., the metabolic data for the different reef flat zones) than rates measured from enclosed organisms or substrates, but all fall within the same order of magnitude.

Open flow respirometry, therefore, may enable determination of the "true" response of a system, because no process or factor is excluded in the measurement procedure. The necessary accompaniment to this approach is relatively large variability in the data. The enclosure technique enables more control over ambient conditions, so that responses directed toward specific external influences can be delineated. It also allows for the required rigor in experimental design, such as independent replication. This approach, ultimately, helps to unravel and explain the more complex patterns seen on the larger scale (Gladfelter 1985).

## Acknowledgments

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RESEARCH

# 16S Mitochondrial Ribosomal RNA Gene Sequences: A Comparison of Seven *Conus* Species

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**The sequences of a 16S rRNA gene segment in mitochondrial DNA from seven *Conus* species were determined. The resulting phylogenetic tree indicates that *Conus californicus*, a non-selective predator is most unrelated to the other *Conus* species. Three putative fish-hunting species were found to be more closely related to each other, despite different shell morphology and geographical distribution. The results are consistent with several lineages of vermivorous *Conus*: the two worm-hunting species, *C. distans* and *C. rattus* do not appear more closely related to each other than to other *Conus* analyzed. The data provide preliminary evidence consistent with the fish-hunting species being more closely related to the snail-hunting *Conus bandanus* than to any of the worm-hunting species.**

Key words: *Conus phylogeny, 16S rRNA, mitochondrial DNA, molecular evolution*

The gastropod genus *Conus* comprises a group of venomous snails that are succcessful predators in tropical marine environments. The genus is relatively young on a geological scale, but has been enormously successful. Despite many attempts to split the genus, no scheme for the division of the cone snails into smaller taxa is generally accepted, and almost all workers have continued to use the single genus *Conus* (Röckel et al. 1995). At the present time, by most standard taxonomic schemes, this is probably the largest genus of living molluscs (ca. 500 species) (Kohn 1956).

*Conus* are clearly distinct from their close relatives, the Terebridae, but their relationship with their closest relatives, the Turridae, is problematic (Taylor et al. 1993). The relationships between cone species are very poorly understood. Most proposed classification schemes are based largely on shell morphology; it is unclear which of the various morphological characteristics used for subdividing the group accurately predict true lineage relationships between cone snail species. On the basis of the major prey envenomated, the cone snails can be divided into three large groups: fish-hunting, snail-

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Abbreviations: dNTP, deoxynucleoside triphosphate; ddNTP, dideoxynucleotide triphosphate; DTT, dithiothreitol;EDTA, ethylenediamine tetraacetic acid; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; TAE, Tris-acetate-EDTA; TE, Tris-EDTA; rRNA, ribosomal RNA

**Table I.** *Conus* species analyzed in this work.

| *Conus* Species | Feeding Type Prey | Locality Collected | Remarks |
|---|---|---|---|
| *C. bandanus* | Gastropod snails | Philippines | |
| *C. californicus* | (Cosmopolitan) | California | Primary diet: polychaetes |
| *C. distans* | Polychaete worms | Philippines | |
| *C. ermineus* | Fish | Caribbean | Hook-and-line strategy |
| *C. ochroleucus* | Fish (?) | Philippines | |
| *C. radiatus* | Fish (?) | Philippines | "Net" strategy (?) |
| *C. rattus* | Polychaete worms | Philippines | |

hunting and polychaete-hunting (vermivorous) species (Kohn 1959). Other phyla (such as echiuroid worms and hemichordates) are prey for at least a few *Conus* species (Kohn 1959). For many hundreds of cone snail species, the biology remains completely undescribed. It has been generally assumed in the literature that the stem line of *Conus* were vermivorous because Turridae prey on polychaete worms, and it is believed that cone snails evolved from a turrid ancestor after the end of the Cretaceous extinction.

Surprisingly, no molecular methods have yet been applied towards assessing evolutionary patterns in *Conus*. Given the impressive advances in molluscan molecular systematics (Adamkewicz et al. 1997, Harasewych et al. 1997), relationships between cone snail species may be more definitively clarified by collecting DNA sequence data to suggest a phylogeny for *Conus*. There are a number of compelling reasons for carrying out such a molecular analysis of *Conus*. Recently, it has been discovered that the venoms of these snails contain remarkable pharmacological agents, with potentially important applications in neuroscience. In certain cases, it is desirable to quickly scan for new *Conus* peptides that may be closely homologous to a peptide already discovered; in other cases, in order to be able to obtain novel ligands that have not yet been discovered, a collection of *Conus* venoms that are as distantly related to each other as possible is required. In the absence of any reliable lineage relationships, making choices of which *Conus* species to investigate is problematic.

In addition, a comprehensive study of the paleontology of cone snails is being carried out by A. Kohn and coworkers (Kohn 1990). It would be timely to provide molecular data to mesh with the fossil evidence. *Conus* is an interesting example of a geologically young marine genus that has undergone several radiations (in the middle Eocene and again in the Miocene), and a number of adaptive specializations have been developed in the course of the history of the genus. A large number of questions can be more cogently addressed with a reliable phylogeny. Did the fish-hunters and snail-hunters evolve from the worm-hunters, or did fish-hunters evolve from the snail-hunting species (or *vice versa*?). Are fish-hunting *Conus* species a monophyletic group, or has fish-hunting evolved multiple times during the history of the genus? How closely related are the major branches of *Conus* species in the Indo-Pacific to the eastern Pacific and Atlantic species?

In this report, we describe an initial attempt to collect molecular data from several *Conus* species of different feeding types. The prey of five of the species used are well-established (see Table I); one is a fish-hunting species (*Conus ermineus*), one a snail-hunting species (*Conus bandanus*) and two are worm-hunters (*Conus rattus* and *distans*). *Conus californicus* is primarily worm-hunting, but it has also been observed to hunt other snails and will even attack fish, although much less efficiently than the fish-hunting specialists. *Conus californicus* is the only species throughout much of its range (i.e., up to central California). It is believed that with no congeners with which to compete, it is one of the few species of *Conus* that preys on multiple phyla. Thus, all major feeding types within the genus are represented in this study. The prey of *Conus radiatus* and *ochroleucus* have not been established, although it has been reported that the closely related species *Conus parius* is fish-hunting (Röckel et al. 1995). *Conus radiatus* and *ochroleucus* appear to be closely related to each other on the basis of shell morphology.

In addition, a broad geographic range is represented. The majority of species are from the Indo-Pacific region, the four specimens being analyzed collected in the Philippines (*Conus ochroleucus, radiatus, bandanus, rattus* and *distans*). *Conus californicus* is from the eastern Pacific marine province, and *Conus ermineus* is from the Atlantic Ocean. Thus, in this initial set of *Conus* to be examined, there is considerable variation in feeding type, geographic range, and shell morphology.

We have obtained and analyzed sequences from 16S ribosomal RNA encoded by the mitochondrial genomes of seven *Conus* species. We demonstrate that the methodology using 16S rRNA gene sequences is feasible to provide molecular data for evaluating the phylogeny of *Conus*. We have also developed and described "universal molluscan 16S rRNA PCR primers" which should be useful tools for the molecular phylogenetic analysis of any molluscan group.

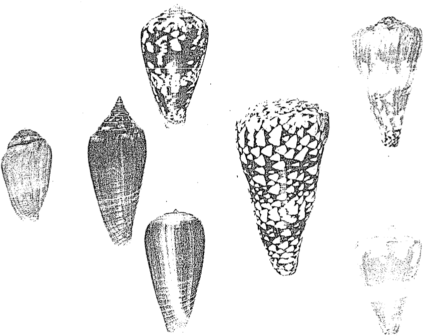

**Figure 1.** Shells of the *Conus* species analyzed. From left to right: *Conus californicus, C. ochroleucus, C. ermineus* (top) and *C. radiatus* (bottom), *C. bandanus*, and *C. distans* (top) and *C. rattus* (bottom).

## Methods

### Specimens

The shells of the seven *Conus* species used were deposited at the University of Utah Museum of Natural History (Accession numbers: *C californicus*, 13781; *C. rattus*, 13782; *C. ermineus*, 13783; *C. distans*, 13784; *C. ochroleucus*, 13785; *C. radiatus*, 13786; *C. bandanus*, 13787).

### Extraction of mitochondrial DNA

DNA was extracted as follows: 30-50 mg of freshly-excised minced hepatopancreas or 1-3 mg of the lyophilized sample was dropped into 1 mL of preheated (10 min at 70° C) extraction buffer (10 mM Tris-HCl pH 8, 2 mM EDTA, 10 mM NaCl, 1% SDS, containing 8 mg DTT and 20 mg RNase A) and incubated for 1 hr with occasional shaking. This was followed by overnight proteinase K digestion (0.4 mg/mL) at 70° C. A 0.5 mL aliquot was purified by phenol/chloroform extraction, then concentrated by centrifugal dialysis using a Millipore Ultrafree-MC filter at 4000 x g for 5-10 min. The quality of the purified DNA was checked by agarose gel (0.5%) electrophoresis.

### Amplification of mtDNA by PCR

For PCR amplification, the set of primers used hybridized to a segment of 16S rRNA gene of cone snails. It consists of an upstream primer 16SL: 5' CGCCTGTTTACCAAAAACAT 3', biotinylated at the 5' end, and a downstream primer 16SH: 5' CCGGTCTGAACTCAGATCACGT 3'. Amplification was carried out in a 10 mL solution with 50 ng of the template DNA, each primer at 0.5 mM, each dNTP at 0.5 mM, 3 mM MgCL$_2$, 50 mM Tris pH 8.3, 0.25 mg/mL BSA and 0.4 unit of *Thermus aquaticus* polymerase (Perkin-Elmer/Cetus). The reaction mixture in a sealed capillary tube was incubated in a rapid air Idaho Technology Air Thermo-Cycler. The PCR profile consisted of an initial denaturation for 2 min at 94° C, followed by 30-40 cycles at the following settings: denaturation for 0 sec at 94° C, annealing for 0 sec at 45-52° C and extension for 15 sec at 72° C. Five microliters of the PCR product was electrophoresed in a 1.5% agarose gel (Seakem, FMC Corp. Rockland, ME) in TAE buffer and the DNA was stained with ethidium bromide. The PCR parameters were then modified depending on the results of the agarose gel analysis.

### Separation of single DNA strand

Twenty microliters of streptavidin-coated magnetic beads (6-7 x $10^8$ beads/mL) (Dynabeads M-280, Dynal, Oslo, Norway) were washed with 20 mL buffer containing 10 mM TrisHCl pH 7.5, 10 mM EDTA and 2 M NaCl. The washed beads were used to capture and immobilize the biotinylated DNA template in 20 mL of the PCR product for 15 min with occasional shaking. The beaded template was isolated from the rest of the solution by magnetic separation (the entire procedure being done at room temperature).

Template denaturation was achieved by incubation of the beaded template with 15 mL of 0.2 N NaOH for 15 min and the beaded single strand DNA was isolated again by magnetic extraction, and sequentially washed with 50 mL 0.2 N NaOH, 40 mL of the Tris-EDTA-NaCl buffer and finally stored as suspension in 50 mL of TE at 4° C. Prior to sequencing, the beads were resuspended in 16 mL H$_2$O.

### Solid phase DNA sequencing

All sequencing reactions were performed using the dideoxy chain termination technique with Sequenase 2.0 DNA Sequencing Kit and/or TAQuence Version 2.0 (United States Biochemicals). The sequences of the two strands of the PCR amplified segment were obtained by using six primers: two primers targeted to the 16S rRNA of the mtDNA of cone snails and other animals (bears), and four internal oligo primers for cone snails (16SL, 5' CGCCTGTTTACCAAAAACAT 3'; 16SH, 5' CCGGTCTGAACTCAGATCACGT 3'; 16LA, 5' CCAGCCTTCAATTATAAGGC 3'; 16LB, 5' GCCTTATA ATTGAAGGCTGG 3'; 16HD, 5' AATGCTGTTATCC CTACGGT 3'; 16HD' 5' ACCGTAGGGATAACAGCATT 3'). The oligonucleotides were synthesized by the University of Utah central facility.

The magnetic bead-bound single strand template from 10 mL of PCR product was annealed to 10 pmol of primer by heating to 70° C for 5 min and cooling slowly to 40° C over a period of about 15 min. The annealed mixture was labeled using 0.5 mL (a-35S)dATP (Amersham, 10 mCi/mL), 1.5 pmol each of dGTP, dCTP and dTTP, and 3.25 U of Sequenase

Version 2.0 T7 DNA polymerase for 3.5-4 min at room temperature. The labeled DNA (3.5 mL) was added to each of 4 wells in a MicroWell MiniTray (Nunc Inc. Naperville, IL) containing 320 pmol of each of the dNTPs and 32 pmol of the appropriate ddNTP. The extension and termination reactions were allowed to proceed for 5 min at 37° C and then stopped following the Sequenase V.2 protocol. Three mL of each reaction mixture was loaded on an 8% polyacrylamide sequencing gel.

The TAQuence Version 2.0 Sequencing Kit was used with certain template-primer sets which did not sequence well with the Sequenase 2.0 Kit. The annealing step in the TAQuence protocol is identical with that in the Sequenase 2.0 described above. However, the labeling reaction was done at 45° C for 5 min using DTaq Version 2 DNA polymerase. The extension and termination reactions were likewise done at elevated temperature of 70-80° C for 5 min. The procedures for stopping the reaction and subsequent fractionation by electrophoresis were identical with that of the Sequenase 2 protocol.

### Computer analysis of nucleotide sequences

The *Conus* nucleotide sequence data are treated as unordered multistate characters, where one state is allowed to be transformed directly into another state (Swofford & Olsen 1990). First, the sequences were aligned using PileUp of GCG or Genetics Computer Group package (Devereux et al. 1984). To maximize alignment of similar portions, the sequences were padded with gaps, with gap weight of 5 and gap length weight of 0.3. Then a multiple sequence alignment of sequences from the seven *Conus* species was made by progressive pairwise alignment. The clustering relationships used to create the alignment were shown by plotting a tree using PileUp Figure. The RNA secondary structures were obtained by Fold and Squiggles of GCG on DNA sequences converted to RNA.

### Phylogenetic trees based on DNA sequences

Phylogenetic trees were constructed using PAUP 3.1 or Phylogenetic Analysis Using Parsimony (Swofford 1991). PAUP is a program for reconstructing phylogenies from discrete-character data employing the principle of maximum parsimony to identify the shortest possible tree(s) or the trees that have the least number of nucleotide replacements. An exhaustive search evaluating 945 unrooted trees retained a single shortest tree (Fig. 4) with a length of 219.

A data base search using FastA and Wordsearch from GCG found similar sequences in two other types of molluscs: the pelycopod *Mytilus edulis*, blue mussel (Hoffman et al. 1992), and eleven sequences from the turritellids (Lieberman et al. 1993). These twelve sequences were used as outgroups to perturb the initial relationships among the *Conus* species. A PAUP bootstrapping or random analysis of 1000 replicate trees from all possible trees was performed on the nineteen taxa set (7 *Conus*, *Mytilus* and 11 turritellids). The resulting consensus tree is shown in Fig. 6.

## Results

### Amplification of 16S rRNA sequence

In this study, a ~513 nucleotide segment of mitochondrial 16S ribosomal RNA was amplified by

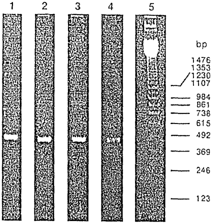

**Figure 2.** Agarose gel electrophoresis of PCR products corresponding to a segment of 16S rRNA gene in mtDNA *Conus* species. The segments were amplified using primers 16SH (biotinylated) and 16SL using conditions described under Methods at varying annealing temperatures. Lanes 1 to 4: *C. distans* (52° C), *C. ochroleucus* (52° C), *C. radiatus* (50° C), *C. californicus* (48° C). Lane 5: 123 bp DNA marker.

```
     1                                                                                        90
Ochr GGCTTCATGT CGAAATCAAA ATATGAAGTC GGACCTGCCC AGTG.AGTTT TAAACGGCCG CGGTACTCTG ACCGTGCAAA GGTAGCATAA
Rad  GGCTTCATGT TAAAATTAAA ATATGAAGTC GGACCTGCCC AGTG.AGTTT TAAACGGCCG CGGTACTCTG ACCGTGCAAA GGTAGCATAA
Ermi GGCTTCGTGT TGAAACTAAA ATACGAAGTC GGACCTGCCC AGTG.AGTTT TAAACGGCCG CGGTACTCTG ACCGTGCAAA GGTAGCATAA
Ban  GGCTTTGTGT TAGTGTTAAG ATACGAAGTC GGACCTGCCC AGTGAATTTT TAAACGGCCG CGGTACTCTG ACCGTGCAAA GGTAGCATAA
Dis  GGCTTCATGT TAAACCTAAA ATATGAAGTC GGACCTGCCC AGTG.AGTTT TAAACGGCCG CGGTACTCTG ACCGTGCAAA GGTAGCATAA
Rat  GGCTTCGTGT CACCTATAA. ATACGAAGTC GGACCTGCCC AGTG.AGTTT TAAACGGCCG CGGTACTCTG ACCGTGCAAA GGTAGCATAA
Cal  GGCTTCATGA CATTTTT.TG ATATGAAGTC GGACCTGCCC AGTGAATTTT TCAACGGCCG CGGTACTCTG ACCGTGCAAA GGTAGCATAA

     91                                                                                      180
Ochr TCATTTGCCT TATAATTGAA GGCTGGAATG AATGGTTTAA CAAGAATACA TCTGTCTCTT TTTGATTATT TAGAATTTTA TTTTTGGATG
Rad  TCATTTGCCT TATAATTGAA GGCTGGAATG AATGGTTTGA CAAGAATACA CCTGTCTCTT TTTGATTGTT TAGAATTTTA TTTTTGGATG
Ermi TCATTTGCCT TATAATTGAA GGCTGGAATG AATGGTTTGA CAAGAATACA CCTGTCTCTT TTAGGCTGCC TAGAATTTTA TCTTTGGATG
Ban  TCATTTGCCT TATAATTGAA GGCTGGAATG AATGGTTTGA CAAGAATACA CCTGTCTCTT TTTGATTGCT TAGAATTTTA TCTCTGGATG
Dis  TCATTTGCCT TATAATTGAA GGCTGGAATG AATGGTTTGA CAAGAATATA ACTGTCTCTC TATGACTACT TAGAATTTTA TTTTTGGATG
Rat  TCATTTGCCT TATAATTGAA GGCTGGAATG AATGGTTTGA CAAGAATACA TCTGTCTCTT TATGATTATT TAGAATTTTA TTTTTAGATG
Cal  TCATTTGCCT TATAATTGAA GGCTGGAATG AATGGTTTGA CAAGAGTGCA ACTGTCTCTT TATGATTCAA TAGAATTTTA TTTTGGGATG

     181                                                                                     270
Ochr AAAAAGTCCA AATATTATTA AAAGACAAGA AGACCCTATC GAGCTTAAGA GAAATTAGTA G.GTTTATAT AAATCAATAT AAGTAAAAGA
Rad  AAAAAGTCCA AATATTATTA AAAGACAAGA AGACCCTATC GAGCTTGAGA GAAATTAGTA G.GCTATTAT AAGTTAATAT AAGTAAAAGA
Ermi AAAAAGTCCA AATATTATTA AAAGACAAGA AGACCCTATC GAGCTTTAGA AAAATTAGTA GACTTAATAT AAATCAATAT AAGTAAAAGG
Ban  AAAAAGTCTA GATATAATTA AAAGACAAGA AGACCCTATC GAGCTTTAGA AGAATTAGTA GATTTAATTT AAATTTATAT AAGTAAAAGA
Dis  AAAAAGTCCA GATTAAATTA AAAGACAAGA AGACCCTATC GAGCTTTAGA GAAATTAGAG GATTTAAATT AATTAATATT TTATAAAAAA
Rat  AAAAAGTCTA GATTTTATTA AAAGACAAGA AGACCCTATC GAGCTTTAAA AAAATTAGCA GGCATACTAG AATTCAATAC CAGTAAAAGA
Cal  AAAAAGTCCT AATATAATTA AAAGACAAGA AGACCCTATC GAGCTTGAAA TAAATTAACA AA...AGAAA AATTTATTAG ACAAAAAAAT

     271                                                                                     360
Ochr AAAGCTATTA AACACTTTGG TTGGGGCAAC CGAGGAGTAA TTAAAGCCTC CTTTAAGTTA GTAAACCTTA CTTGTGTTGA TCCCAAATTT
Rad  AAAACTATTA AATACTTTGG TTGGGGCAAC CGAGGAGTAA CTAGAGCCTC CTTTAAATTA ATAAATCTTA CTTGTGTCGA TCCCAAA.TT
Ermi AAAACTACTA AATACTTTGG TTGGGGCAAC CGAGGAGTAA ATAGAGCCTC CTTTGAATTA CAAATCCTAC ATGTAATTGA TCCCAAA.AT
Ban  AAAACTATTA AACACTTTGG TTGGGGCAAC CGAGGAGTAA ATAAAGCCTC CTTTAGAATA ATAAATCTTA CTTGTGTTGA TCCCAAA.TT
Dis  TAGACTTCTA TAAACATTGG TTGGGGCAAC CTGGGAGTAT ATAAAGCCTC CTTTAATTGT ATAAGTCTTA CAAGTATTGA TCC.AAACTT
Rat  AAGGCTGTTA AATATTTTGG TTGGGGCAAC CTAGGAGTAA GTAAAGCCTC C.TAACTACA GTAAGTCGGA CTTGTGTTGA TCC.AGGTAT
Cal  TGTGTTGTTA AACATTTTGG TTGGGGCAAC TAAGGAGTAA AAAAAGCCTC CTTTATGTTA AGATAAACTA ACAAGTACTG ATCGAACTT

     361                                                                                     450
Ochr TTTGATCAAA GGAATTAGTT ACCGTAGGGA TAACAGCATT ATCTTTTTTA AGAGCCCATA TCGAAAAAAA GGTTTGTGAC CTCGATGTTG
Rad  TTTGATCAAA GGAATTAGTT ACCGTAGGGA TAACAGCATT ATCTTTTTTA AGAGCCCATA TCGAAAAAAA GGTTTGTGAC CTCGATGTTG
Ermi TTTGATCAAA GGAATTAGTT ACCGTAGGGA TAACAGCATT ATCTTTTTCA AGAGCCCATA TCGAAAAAAA GGTTTGTGAC CTCGATGTTG
Ban  TTTGATCAAA GGAATTAGTT ACCGTAGGGA TAACAGCATT ATCTTTTTTG AGAGCTCATA TCGAAAAAAA GGTTTGTGAC CTCGATGTTG
Dis  TTTGATCAAA GGAATTAGTT ACCGTA.GGA TAACAGCATT ATCTTTTTTG AGAGCCCATA TCGAAAAAAA GGTTTGTGAC CTCGATGTTG
Rat  AATGATCAAA GGAATTAGTT ACCGTAGGGA TAACAGCATT ATCTTTTTTG AGAGCCCATA TCGAAAAAAA GGTTTGTGAC CTCGATGTTG
Cal  TTTGATCAAA GAAAATAGTT ACCGTAGGGA TAACAGCATT ATCTTTTTTG AGAGTTCCTA TCGAAAAAAA GGTTTGTGAC CTCGATGTTG

     451                                                          513
Ochr GACCAGAATA TCCTAAAGAT GCAGAAGTCT TTAAGGGTTG GTCTGTTCGA CCATTAAAAT TCT
Rad  GACCAGAATG TCCTAAAGAT GCAGAAGTCT TTAAGGGTTG GTCTGTTCGA CCATTAAAAT TCT
Ermi GACCAGAATA TCCTGAAGAT GCAGAAGTCT TTAAGGGTTG GTCTGTTCGA CCATTAAAAT TCT
Ban  GACCAGAATG TCCTAAAGAT GCAGAAGTCT TTAAGGGTTG GTCTGTTCGA CCATTAAAAT TCT
Dis  GACCAGAATG TCCTGAAGAT GCAGAAGTCT TCAAGGGTTG GTCTGTTCGA CCATTAAAAT TCT
Rat  GACCAGAATG TCCTAAAGAT GCAGAAGTCT TTGAGGGTTG GTCTGTTCGA CCATTAAAAT TCT
Cal  GACCAGAATA TCCTGAAGAT GCAGAAGTCT TCAAGGGTTG GTCTGTTCGA CCATTAAAAT TCT
```

**Figure 3.** Aligned DNA sequences of a segment of mitochondrial 16S rRNA gene from seven *Conus* species. The GenBank accession numbers of the nucleotide sequences are: *C. ochroleucus*, AF036528; *C. radiatus*, AF036529; *C. ermineus*, AF036530; *C. bandanus*, AF036531; *C. distans*, AF036532; *C. rattus*, AF036533; and *C. californicus*, AF036534. Sequences were aligned using PileUp of GCG with gaps given a weight of 5 and gap length weight of 0.3. Gaps are indicated as dots.

PCR (see Fig. 2) using *Conus*-specific primers obtained by preliminary sequencing of six species. The optimum conditions for PCR amplification with these primers varied among the seven species of cone snails. Optimum stringency conditions were obtained by varying either the annealing temperature, the number of cycles, enzyme concentration or a combination of these factors.

### Comparison of sequences of *Conus* species

The sequences obtained from the PCR amplification product are shown in Fig. 3. Both strands were sequenced, and in addition to the original PCR primers, several internal primers described in Methods were used for sequencing. A solid phase sequencing protocol was used for most of these studies.

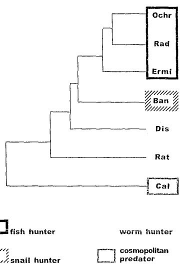

**Figure 4.** Cladogram of seven *Conus* species *C. ochroleucus*, *C. radiatus*, *C. ermineus*, *C. bandanus*, *C. distans*, *C. rattus*, *C. californicus*) generated by parsimony analysis (PAUP) of a 513-bp segment of the 16S rRNA gene of mitochondrial DNA.

The sequences are aligned to yield maximal identity (Fig. 3). Of the 513 nucleotides in most sequences, approximately 25% (132 out of the 513) have nucleotide substitutions for at least one of the *Conus* species analyzed. It is noteworthy that the variable regions are highly clustered; thus, from position 322 to 362, 90% of all loci exhibit variability; in contrast, from position 53 to 138, every locus shows the same nucleotide in all seven species.

### Phylogenetic trees based on DNA sequences

The DNA sequences in Fig. 3 were used to construct phylogenetic trees using the GCG and PAUP programs discussed under Methods. All of the trees generated immediately reveal that one species, *Conus californicus*, is much more distantly related to the other *Conus* species examined (for example, see Fig. 4). This does not appear to be because of its geographic isolation; *Conus ermineus*, which is found in the Atlantic marine province is more closely related to *Conus radiatus* and *ochroleucus* than to any other *Conus* species. The close relationship of *Conus radiatus* and *ochroleucus* was, in part, expected because these species are sometimes mistaken for each other.

The *Conus* DNA sequences were also compared with the corresponding sequences previously published for two groups of molluscs: the pelycopod *Mytilus edulis*, blue mussel (Hoffman et al. 1992) and a group of gastropods in the family *Turritellidae* which belong to the same subclass (*Prosobranchia*) as the *Conus* family. Lieberman et al. (Lieberman et al. 1993) reported DNA sequences from segments of the 16S rRNA encoded by mitochondrial genome from eleven species in four genera representing two families: the *Potamididae* (Adams and Adams, 1854) and the *Turritellidae* (Loven, 1847). These turritellid sequences aligned with the 3' portion of the *Conus* sequences from nucleotide position 228 to 495 (in a total span of 513 bp).

The turritellid sequence length of 271 bp shares 42% homology with the *Conus* set, whereas *M. edulis* sequence shows 38% identity with the cone snails in this region (Fig. 5). However, the aligned sequences in the same region from all three groups of molluscs give 74 identical nucleotides out of 271 or only 27% identity. The eleven turritellid sequences and that of the *M. edulis* sequence were used as outgroups. The bootstrap option of the parsimony method PAUP was used for evaluating 1000 replicate trees sampled from the set of all possible random trees generated by nineteen taxa (7 cones, a mussel and 11 turritellids). The minimum length tree for the all-*Conus* set and that with the outgroups showed similar tree topology. Both trees show that the fish hunters, *C. ermineus*, *C. ochroleucus* and *C. radiatus*, fall in the same clade although their relative positions within the clade differ. All trees show that *C. californicus* is the most distantly related. However, the two figures show a discrepancy in the relative positions of the two worm hunters, *C. distancs* and *C. rattus*, when the tree is pertubed by the outgroup species, probably indicating a need for using a more closely related outgroup set. Bootstrap analysis of the 1000 randomly sampled trees shows that almost all the nodes are supported by 100% (see Fig. 6). Moreover, 75% of the replicate trees

```
        1                                                                                  90
Msl     ccctatgaag ctttatctta attggagctc tc........ .......... ........ag gctcttatac gattttgatg ggagatcagt
Rat     CCCTATCGAG CTTTAAAAAA ATTAGCAGGC ATACTAGAAT TCAATACCAG TAAAAGAAAG GCTGTTAAAT ATTTTGGTTG GGGCAACCTA
Ochr    CCCTATCGAG CTTAAGAGAA ATTAGTAG.G TTTATATAAA TCAATATAAG TAAAAGAAAA GCTATTAAAC ACTTTGGTTG GGGCAACCGA
Rad     CCCTATCGAG CTTGAGAGAA ATTAGTAG.G CTATTATAAG TTAATATAAG TAAAAGAAAA ACTATTAAAT ACTTTGGTTG GGGCAACCGA
Ermi    CCCTATCGAG CTTTAGAAAA ATTAGTAGAC TTAATATAAA TCAATATAAG TAAAAGGAAA ACTACTAAAT ACTTTGGTTG GGGCAACCGA
Ban     CCCTATCGAG CTTTAGAAGA ATTAGTAGAT TTAATTTAAA TTTATATAAG TAAAAGAAAA ACTATTAAAC ACTTTGGTTG GGGCAACCGA
Dis     CCCTATCGAG CTTTAGAGAA ATTAGAGGAT TTAAATTAAT TAATATTTTA TAAAAAATAG ACTTCTATAA ACATTGGTTG GGGCAACCTG
Cal     CCCTATCGAG CTTGAAATAA ATTAACAAA. ..AGAAAAAT TTATTAGACA AAAAAATTGT GTTGTTAAAC ATTTTGGTTG GGGCAACTAA
Ced     ccctgtcgag ctt..aaaga accgtagaa. gtttatatag attttaaata aataatcta. ..t.tgagct ttttagttgg ggcga.ccaa
Tuh     ccctgtcgag cttaaaaagt aagttagaa. atttatatat gatctcaaat aaaattcta. ..t.tgttat ttttggttgg ggcga.ccaa
Mrc     ccctgtcgag cttgaaaaac aaacagaaac acttatgttt tatagcaaat atataactg. ..t.taatct ttttggttgg gggcgaccaa
Vkn     ccctgtcgag cttaaaagcc aacagataac acctatatta ttta..taat atataacta. ..t.taacct ttttggttgg gggcgaccga
Tua     ccctgtcgag cttgaaaaac aagagaagct atcttatatt ttaattaaat tcataactg. ..c.tagtat ttttggttgg ggcgacccaa
Tug     ccctgtcgag cttgaagggt aagtagaagc a.attatatt ttaattaaat ttacagcta. ..c.taatac ttttggttgg ggcga.ccaa
Tub     ccctgtcgag cttaaaaaat aaatag..aa ctacttatat tttaataaat ttacagcta. ..t.cagtat ttttggttgg ggcgaccgag
Tue     ccctgtcgag cttaaaaaat aaataggaag ccacttatat tttaataaat ttacagcta. ..t.tagtat ttttggttgg ggcgaccgag
Tuc     .cctgtcgag ctttaaaaat aaatagaaac atttataca. ..tagtaaat tttcaacta. ..t.tatttt ttttggttgg ggcgaccaag
Tuf     ccctgtcgag cttaaaaaat ggatggaaag a..tttatat tttaatagat tcatagcta. ..t.tcggtg ttttggttgg gggcgtcccg
Tud     ccctgtcgag cttaaaaaaa cgagcgggaa caacctatac tctatgctaa atatatcaac csy.taaatt ttttggttgg gggcgaccaa

        91                                                                                 180
Msl     agaaataagt cttctactat catattaatc t......... .....tacta gtatttccta actttatatg tgtggctagc tactctaggg
Rat     GGAGTAAGTA AAGCCTCCTA ACTA.CAGTA ....AGTCGG ACTTGTGTTG ATCC.AGGTA TAATGATCAA AGGAATTAGT TACCGTAGGG
Ochr    GGAGTAATTA AAGCCTCCTT TAAGTTAGTA ....AACCTT ACTTGTGTTG ATCCAAAATT TTTTGATCAA AGGAATTAGT TACCGTAGGG
Rad     GGAGTAACTA GAGCCTCCTT TAAATTAATA ....AATCTT ACTTGTGTCG ATCCAAAA.T TTTTGATCAA AGGAATTAGT TACCGTAGGG
Ermi    GGAGTAAATA GAGCCTCCTT TGAATTACAA ....ATCCTA CATGTAATTG ATCCAAAA.A TTTTGATCAA AGGAATTAGT TACCGTAGGG
Ban     GGAGTAAATA AAGCCTCCTT TAGAATAATA ....AATCTT ACTTGTGTTG ATCCAAAA.T TTTTGATCAA AGGAATTAGT TACCGTAGGG
Dis     GGAGTATATA AAGCCTCCTT TAATTTTATA ....AGTCTT ACAACTATTG ATCC.AAACT TTTTGATCAA AGGAATTAGT TACCGTA.GG
Cal     GGAGTAAAAA AAGCCTCCTT TATGTTAAGA ....TAAACT AACAAGTACT GATCCAAACT TTTTGATCAA AGAAAATAGT TACCGTAGGG
Ced     ggaacaaaaa aagcttctta aatataaaaa ......actt acaagtttag atcc.aatta gtttgattaa agaaattagt taccgcaggg
Tuh     ggaacaaaaa aagcttcctt atttttcttg ......acct atatgttttg atccggctaa agctgactaa aaaaattagt taccgcaggg
Mrc     ggaacaaaaa gagcttcctt tataaattaa tttaaaacct atacgttttg atccagc.aa agctgattaa aaaaactagt taccgcaggg
Vkn     ggaacaaaaa aagcttcctc t.....taac ttataaacct atttgttttg atccagctaa agctgattaa aaaaactagt taccgcaggg
Tua     ggaacaaaaa aagcttcctg tattaagaaa t.....accg ataagttttg atccggct.t agccgattaa aagaattagt taccgcaggg
Tug     ggaacaaaaa aagcttcctg tatttaagta t.....acta ataagttttg atccagct.t agctgattaa aaaaattagt taccgcaggg
Tub     g.aacaaaaa aagcttccta tattttaaaa t.....acta atgagtcttg atgc..ccaa agccgattaa aaaaattagt taccgcaggg
Tue     gaaacaaaaa aagcttccta tacttaataa t.....acta atgagtcctg atccagccaa agctgattaa aaaaattagt taccgcaggg
Tuc     g.aacaacaa aagcttcctc tattcttttt t.....actt ataagttttg atccag.taa aactgatcaa aaaaattagt taccgcaggg
Tuf     ggaacaagaa aagcttcct. ....tagttc tgggggaccg gttagttttg acccagc.aa agctgaccaa tagaattagt taccgcaggg
Tud     ggaacaaaag aagcttcctc tatatactaa ttcaascata tttgttctga tccagtgtaa aactgattaa aagaactagt taccgcaggg

        181                                                                                  271
Msl     ataacagcgc aatttctccc gaaagatggt attggagggg aagattgcga cctcgatgtt ggctttagat atcctagagg cggagaagct t
Rat     ATAACAGCAT TATCTTTTTT GAGAGCCCAT ATCGAAAAAA AGGTTTGTGA CCTCGATGTT GGACCAGAAT GTCCTAAAGA TGCAGAAGTC T
Ochr    ATAACAGCAT TATCTTTTTT AAGAGCCCAT ATCGAAAAAA AGGTTTGTGA CCTCGATGTT GGACCAGAAT ATCCTAAAGA TGCAGAAGTC T
Rad     ATAACAGCAT TATCTTTTTT AAGAGCCCAT ATCGAAAAAA AGGTTTGTGA CCTCGATGTT GGACCAGAAT GTCCTAAAGA TGCAGAAGTC T
Ermi    ATAACAGCAT TATCTTTTTC AAGAGCCCAT ATCGAAAAAA AGGTTTGTGA CCTCGATGTT GGACCAGAAT ATCCTGAAGA TGCAGAAGTC T
Ban     ATAACAGCAT TATCTTTTTT GAGAGCTCAT ATCGAAAAAA AGGTTTGTGA CCTCGATGTT GGACCAGAAT GTCCTAAAGA TGCAGAAGTC T
Dis     ATAACAGCAT TATCTTTTTT GAGAGCCCAT ATCGAAAAAA AGGTTTGTGA CCTCGATGTT GGACCAGAAT GTCCTGAAGA TGCAGAAGTC T
Cal     ATAACAGCAT TATCTTTTTT GAGAGTTCCT ATCGAAAAAA AGGTTTGTGA CCTCGATGTT GGACCAGAAT ATCCTGAAGA TGCAGAAGTC T
Ced     ataacagcat aatctctctt gagagaccta atcgaaaggg ggggttgtga cctcgatgtt ggactagnat atcctaaaga tgcagaagtc t
Tuh     ataacagcat aatctctctt aagagaccta atcgaaagag ggggttgtga cctcgatgtt ggactagaat atcctaaaga tgcagaagtc t
Mrc     ataacagcat aatctccctt gagagaccaa atcgaaaggg ggggttgtga cctcgatgtt ggactagaat atcctaaaga tgcagaagtc t
Vkn     ataacagcat aatctccctt gagagaccaa atcgaaaggg ggggttgtga cctcgatgtt ggactagnat atcctaaaga tgcagaagtc t
Tua     ataacagnat aatctctctt gagagaccta atcgaaaggg ggggttgtga cctcgatgtt ggactagnat atcctgaaga tgcagaagtc t
Tug     ataacagaat aatctctctt gaga.cccta atcgaaaggg ggggttgtga cctcgatgtt ggactagnat atcctaaaga tgcagaagtc t
Tub     ataacagcat aatctccctt gagagacctt atcgaaaggg ggggttgtga cctcgatgtt ggactagnat atcctaaaga tgcagaagtc t
Tue     ataacagcat aatctccctt gagagacctt atcgaaaggg gggattgtga cctcgatgtt ggactagaat atcctgaaga tgcagaagtc .
Tuc     ataacagcat aatctccttt gagagaccat atcgaaaggg ggggttgtga cctcgatgtt ggactagaat atccggaaga tgcagaagtc t
Tuf     ataacagcat gatctccctt gagaggcccc atcgaaaggg ggggttgtga cctcgatgtt ggattaggat atcctgaaga tgcagaagtc t
Tud     ataacagcat aatctccttt gagagaccaa atcgaaaggg ggggttgtga cctcgatgtt ggactaggat atcctaaaga tgcagaagtc t
```

**Figure 5.** Aligned DNA sequences of part of the mitochondrial 16S rRNA gene from seven *Conus* species (*C. ochroleucus, C. radiatus, C. ermineus, C. bandanus, C. distans, C. rattus, C. californicus*), *Mytilus edulis* (Msl) and eleven turritellids. Sequences were aligned using PileUp of GCG with gaps given a weight of 5 and gap length weight of 0.3. Gaps are indicated as dots.

support the idea that the fish-hunters belong to the same clade. All trees consistently indicate that *C. californicus* is the most divergent of the *Conus* species; as expected, the *M. edulis* sequence is the most unrelated when all nineteen taxa are considered.

### Implications for *Conus* taxonomy

The seven species chosen for analysis represent different feeding types, different morphologies within the genus and different geographic distribution. We have deliberately analyzed two species that, by all criteria, are closely related (*Conus radiatus* and *Conus*

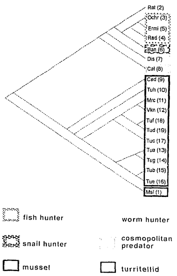

**Figure 6.** Consensus tree showing the relationships of seven *Conus* species (*C. ochroleucus, C. radiatus, C. ermineus, C. bandanus, C. distans, C. rattus, C. californicus*), *Mytilus edulis* and eleven turritellids.

*ochroleucus*) and also included species that are distant as possible in every possible respect. Some of the species are found over a vast geographic region (i.e., *Conus distans, bandanus* and *rattus*) while others are much more narrowly distributed geographically (*Conus californicus* and *Conus ochroleucus*).

## Discussion

The results of the preliminary molecular analysis of an ~513 base mitochondrial ribosomal RNA gene segment from these seven different *Conus* species indicate that using this as a data set for clarifying phylogenic relationships in *Conus* is quite feasible. The results reveal that of the seven species, *Conus californicus* is most distantly related to the others. The results also suggest that three species, *Conus ermineus, radiatus* and *ochroleucus* are more closely related to each other than they are to any of the other *Conus* species analyzed. On the basis of shell morphology alone, *Conus ermineus* would not have been considered closely related to either *C. radiatus* or *C. ochroleucus*. Nevertheless, the mitochondrial DNA sequence data indicate that *Conus ermineus* is more closely related to *Conus ochroleucus* and *radiatus* than to any of the other species. If *Conus radiatus* and *ochroleucus* are indeed fish-hunting, this suggests that fish-hunting *Conus* species may comprise a species clade within the genus. This suggestion clearly needs to be confirmed by a much more extensive analysis of fish-hunting species.

Almost all proposed subgeneric classifications have put *Conus ochroleucus* and *radiatus* in one subgenus, and *Conus ermineus* in another. For example, in a recent comprehensive systematic classification proposed largely on the basis of shell morphology (da Motta 1991), *Conus radiatus* was assigned to the genus *Hermes* (Montfort, 1852), and designated the type species of subgenus *Phasmoconus* (Mörch, 1852), one of eight proposed subgenera in *Hermes*. In contrast, *Conus ermineus* was assigned to the genus *Dendroconus* (Swainson, 1940), and designated type of the subgenus *Chelyconus* (Mörch, 1952). This proposed classification would have put *Conus californicus* in *Dendroconus*. The molecular data are clearly inconsistent with such a taxonomic scheme for these three species (*C. ermineus, radiatus* and *californicus*). Thus, the molecular data obtained in this study can be used to evaluate such taxonomic proposals.

Thus, the data suggest that some species that seem to be closely-related by shell morphology may in fact be evolutionarily quite distant (i.e., *Conus californicus* and *ermineus*), and vice-versa (*Conus ochroleucus* and *ermineus*). When an evolutionary barrier is overcome (such as colonizing a new prey type), there could be a rapid radiation of species which may result in very rapid shell morphological diversification within the new clade of species.

The results are suggestive of several distinct lineages of worm-hunting cones since *Conus distans* and *rattus* do not appear to be more closely related to each other than they are to the other *Conus* in the group. Although *Conus californicus* is primarily a

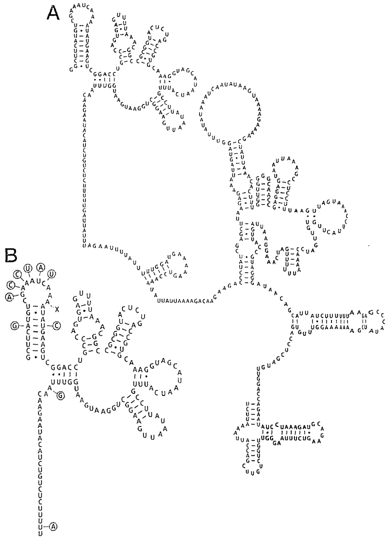

Figure 7. The nucleotide sequence of *C. ochroleucus* was folded as described under Methods (see A). The substitutions at the 5' end of the sequence in *C. rattus* are indicated in panel B. Note that most are concentrated in the first loop, and that the stem substitutions retain the base pairing.

worm-hunting species, it clearly is phylogenetically the most distant, in concordance with the previous suggestion that *Conus californicus* may belong to the most ancient lineage of *Conus* (Kohn 1990).

The precise placement of the other *Conus* species is somewhat uncertain, but the data are suggestive that the fish-hunting cones and snail-hunting cones may have a common phyletic origin. The preliminary scheme proposed here which shows that the snail-hunting, fish-hunting and one clade of worm-hunting *Conus* species all arose from a common ancestor remains to be confirmed by a more detailed analysis using additional DNA sequences, and a more extensive sampling of *Conus* species.

**Greater variability in selected loop regions.** When 16S ribosomal RNA sequences from two different *Conus* species are compared, the distribution of substitutions is highly non-random. The results provide strong evidence that a subset of loops of stem-loop structures are much more variable than the average sequence. The most striking example is detailed in Fig. 7 and Table II. The figure shows a proposed secondary structure for residues 1 to 128 of the 16S rRNA segment shown in Fig. 2 for *Conus ochroleucus*. It will be noted that there are five distinct stem-loop structures. We found that the loop in the first of these (residues 11 to 20) is the most hypervariable region in the entire sequence of 513 residues examined in this study. In contrast, the other residues in region 1 to 128 are less variable on the average than the sequence as a whole (which is not surprising since from residue 53 on, the region is invariant). If a pair-wise comparison is made between the sequence shown in Fig. 7 and each of the six other sequences obtained from the *Conus* species, between 30-80% of the nucleotides in this loop are found to be substituted. If this loop is excluded, the rest of the region (residues 1 to 128) only shows a substitution frequency of <0.1% to 4.2%, which in every case is less variable than the average substitution frequency for the entire sequence of 513 nucleotides (see data in Table II). Thus, the frequency of nucleotide substitution can vary >300-fold in different regions.

In addition to the variable loop in residues 11-20, the putative loops found in 241-264, 307-317, 327-342 and 356-360 also exhibit an excess in substitution frequency compared to the average rate for the entire 513 nucleotide region. It should be noted that not all loop regions are variable; the other loops besides 11-20 shown in Fig. 7 are mostly invariant.

The feasibility of using 16S ribosomal RNA sequence as a probe in molluscan taxonomy is demonstrated.

The work we have described here constitutes a feasibility study to determine whether a straightforward PCR approach could be used to assess the evolutionary relationships between *Conus* species. We believe that these initial results indicate that the approach of using the 16S ribosomal RNA may be useful not just for *Conus* taxonomy but for molluscan taxonomy in general. Part of the reason for this optimism is that the presence of both highly variable regions and extremely conserved ones (as well as many with intermediate variability) makes this RNA a particularly flexible tool for molecular taxonomy. The mutation rate in the highly variable regions can be used to provide insight into the

**Table II.** Substitution Frequency (Reference: *Conus ochroleuscus* sequence)

| Species | Whole Segment | | 5' end of segment (1-128) | | | |
|---|---|---|---|---|---|---|
| | | | Hypervariable loop | | w/o Hypervariable loop | |
| | (1-513) | (%) | (11-20) | (%) | (#) | (%) |
| C. *radiatus* | 21 | 4.1 | 3 | 30 | 0 | <0.1 |
| C. *ermineus* | 40 | 7.8 | 3 | 30 | 2 | 1.7 |
| C. *bandanus* | 41 | 8.0 | 7 | 70 | 5 | 4.2 |
| C. *rattus* | 53 | 10.3 | 7 | 70 | 2 | 1.7 |
| C. *distans* | 60 | 11.7 | 5 | 50 | 0 | <0.1 |
| C. *californicus* | 70 | 13.6 | 8 | 80 | 4 | 3.4 |
| Total nucleotides in regions | 513 | | 10 | | 118 | |

**Table III.** Proposed PCR primers derived from conserved mtDNA sequences

| Primer | Sequence | Base Position (*Conus* L strand) | Conserved In |
|---|---|---|---|
| 16LC | 5' GTTTACCAAAAACATGGCTTC 3' | ( – 15) – 6 | C |
| 16HC | 5' AGAATTTTAATGGTCGAACAG 3' | 513 – 493 | C |
| 16LT1 | 5' AATTGAAGGCTGGAATGAA 3' | 118 – 137 | C |
| 16LT2 | A T | | M |
| 16HM1 | 5' CCAACATCGAGGTCACAAAC 3' | 465 – 446 | C, T |
| 16HM2 | G T | | M |

C = *Conus* snail, T= turritellids, M = *Mytilus edulis*

taxonomy of closely-related species within a genus (such as *Conus radiatus* and *ochroleucus* among the species examined), while the intermediate and conserved regions in relatively short stretches of DNA can be used to explore more distant relationships. Thus, the relatively small sequence region for which sequences for *Conus*, turitellids and *Mytilus* are all available yielded a data set useful for suggesting what appears to be a quite sensible taxonomic scheme. Clearly the trees in Figs. 4 and 6 only provide an initial hypothesis which needs to be refined and tested by additional morphological, molecular, paleontological and biological data.

Furthermore, because there are extremely constant regions, it is possible to use these for the design of probes that should work for all molluscan groups. Shown in Table III are our proposed "universal" molluscan primers, which are conserved between the seven *Conus* species and *Mytilus*; given the extreme evolutionary distance between these, the probes seem likely to work for every molluscan group. We are currently testing the new probes on additional *Conus* species, and evaluating the relationships between the cone snails and the turrids. We will be pleased to provide these universal molluscan PCR primers to anyone interested in using them for investigating other molluscan groups.

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# Multiple Bromotryptophan and γ-carboxyglutamate Residues in a *Conus* Peptide

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**A novel peptide was purified from *Conus textile* venom which caused hyperactivity in mice. The 31-amino acid peptide has six residues with unusual post-translational modifications: four γ-carboxyglutamates and two brominated tryptophan residues. This peptide, which we have designated the dibromorunning peptide, is the first known gene product with multiple bromotryptophan residues. We discuss the apparent non-random association of γ-carboxyglutamate and bromotryptophan in *Conus* peptides.**

**Key words:** *Conotoxin, bromotryptophan, γ-carboxyglutamate, hyperactivity, Conus textile, post-translational modification*

The predatory cone snails use a peptide-based neuropharmacological strategy to capture prey, defend against predators and interact with competitors (Olivera 1997). Approximately 100 different peptides can be found in the venom of each *Conus* species. Furthermore, the peptides made by one species are a distinctive set, different from the peptides in any other venom. Since there are approximately 500 different species of cone snails, ca. 50,000 different peptides are likely present in the venoms of the living *Conus*. Only a tiny fraction of these have been characterized, even superficially.

The conopeptides are mostly small (8 - 35 amino acids in length) and multiply disulfide-bonded (Olivera 1997, Olivera et al. 1990). One of the hallmark characteristics of a significant proportion of *Conus* peptides is the unusual post-translational modifications found, some previously undescribed. Among the most distinctive are the γ-carboxylation of glutamate residues to yield γ-carboxyglutamate (Gla) (McIntosh et al. 1984) and the bromination of tryptophan residues to 6-bromotryptophan (Trp) (Craig et al. 1997; Jimenez et al. 1997). Although these post-translational modifications are unusual even among *Conus* peptides, γ-carboxyglutamate and bromotryptophan were found together in two previously characterized peptides. The first was the bromosleeper peptide from *Conus radiatus* (Craig et al. 1997). More recently, a peptide belonging to a new superfamily of *Conus* toxins, tx5a of the T-superfamily, was found to have both 6-bromotryptophan and γ-carboxyglutamate (as well as an O-glycosylated threonine residue) (Rigby et al. 1999, Walker et al. 1999).

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Abbreviations: DTT, dithiothreitol; ESI, electrospray ionization; Gla, γ-carboxyglutamate; HPLC, high performance liquid chromatography; Hyp, hydroxyproline; PTH, phenylthiohydantoin; TFA, trifluoroacetic acid; Trp, bromotryptophan.

In this report, we describe a novel peptide from *Conus textile* venom which is the third to have both these two unusual post-translational modifications. This peptide that we refer to as dibromorunning peptide or tx7a has three disulfide linkages, four Gla residues and, uniquely, two residues of bromotryptophan.

## Methods

### Purification of the peptide

Lyophilized *Conus textile* venom from the Philippines (200 mg) was extracted sequentially with 10 mL each of 0%, 20%, 40% and 60% acetonitrile. The mixture was sonicated for 30 s periods over ice, centrifuged at 5,000 x g for 5 min and the combined supernatant was stored at -20° C. Aliquots of the extract were fractionated by HPLC chromatography on a $C_{18}$ Microsorb MV analytical column or a Vydac $C_{18}$ semi-preparative column as previously described (Walker et al. 1999). The columns were eluted with a 0.45%/min gradient of acetonitrile ($CH_3CN$) in 0.1% trifluoroacetic acid (TFA) at 1 mL/min for the analytical column or 5 mL/min for the semi-preparative column (Fig. 1). Corresponding peaks from several runs were pooled and peptide tx7a was purified from the pool by two consecutive runs on the analytical column eluted with 0.23%/min of $CH_3CN$ in 0.1% TFA. The final purification was a run on the same column eluted isocratically at 38% $CH_3CN$ in 0.1% TFA.

### Bioassay

The biological activity of HPLC fractions was determined by intracranial (i.c.) injection into 9- to 15-day-old mice. Fractions were lyophilized then dissolved in normal saline solution before injection using an ultrafine insulin syringe. The 26-gauge needle was allowed to penetrate the cranium to ~1.0-1.5 mm depth. Control mice were injected with equal volume (15-20 μL) of normal saline solution containing dissolved residue (if any) of lyophilized column buffer. After injection, the mice were returned to their cages and observed for any behavioral symptoms. The time at which the mouse first showed abnormal behavior was also recorded. The dose used was ~0.3 to 0.5 nmole per mouse. A higher dose was not tried due to a limited amount of sample.

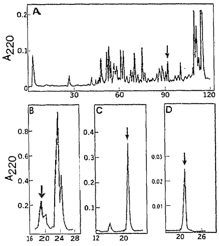

Figure 1. Purification of the dibromorunning peptide, tx7a, from *C. textile* venom. (A) HPLC chromatogram of an aliquot of the crude venom extract obtained as described under Methods. (B) A pool of fractions corresponding to the bioactive peak indicated by arrow in Panel A was applied to an analytical $C_{18}$ Microsorb MV column, and then eluted with a 0.23%/min gradient of 38-48% acetonitrile. (C) The bioactive fraction in Panel B was repurified using the same conditions in B. (D) Isocratic elution of the pure peptide with 38% acetonitrile. The bioactive peak in panels B through D is indicated by an arrow.

### Reduction and alkylation

The purified peptide was reduced with dithiothreitol (DTT) and alkylated with 4-vinylpyridine as follows. The peptide solution was adjusted to pH 8 with 0.5 M TRIS before DTT was added to a concentration of 10 mM. The solution was flushed with $N_2$, incubated at 65° C for 15 min then cooled to room temperature. After adding 4-vinylpyridine (5 μL per mL of solution), the mixture was left in the dark at room temperature for 25 min. The mixture was diluted with 0.5 mL of 0.1%

TFA prior to purification of the reduced peptide by HPLC with the $C_{18}$ Microsorb MV analytical column.

### Digestion of the peptide

Approximately 1.5 nmol of peptide was digested with endoproteinase AspN (Boeringer Mannheim). The lyophilized peptide was dissolved in 0.1mL of 50 mM sodium phosphate, pH 8. Endoproteinase AspN (1 mg enzyme per 20 mg peptide) in 10 mM TRIS Cl, pH 7.5, was added, and the reaction incubated for 17 h at 37° C. To stop the reaction, 0.1% TFA (0.5 mL) was added, and the digest was fractionated by HPLC with a $C_{18}$ Microsorb MV analytical column eluted with a linear gradient of 0.9% acetonitrile per mL/min. The intact mass of the digestion fragments were analyzed by ESIMS prior to chemical sequencing.

### Mass spectrometry

Electrospray ionization (ESI) mass spectra were measured using an Esquire ion trap mass spectrometer (Bruker Daltonics, Billerica, MA). The HPLC-purified sample, collected in 0.1% TFA and acetonitrile was diluted in methanol-1% acetic acid, transferred to a fused silica capillary and infused at approximately 500 nL/min. The mass accuracy of the ion trap instrument was typically better than 200 ppm.

### Peptide sequencing

The peptide was reduced with DTT and alkylated with 4-vinylpyridine. Approximately 10 to 100 pmol of alkylated peptide and N-terminal digestion fraction fragments were sequenced by Edman degradation with an Applied Biosystem Model 492 Sequenator (DNA Peptide Facility, University of Utah). The 3-phenol-2-thiohydantoin derivatives were identified by HPLC. Predicted masses for each sequence were routinely verified by mass spectrometry.

### Identification and characterization of a cDNA

The Expressed Sequence Tag (EST) method previously described (Walker et al. 1999) was used to identify a cDNA clone encoding tx7a. Templates were prepared using a QIAprep Spin Miniprep Kit (QIAGEN, Valencia, CA) and submitted for fluorescent sequencing primed with nucleotides, M13R and subsequently M13U (Messing 1983) at the Health Sciences Center Sequencing Facility, Eccles Institute for Human Genetics, University of Utah. All molecular biology techniques were as described in Sambrook, Fritsch and Maniatis (Sambrook et al. 1989).

## Results

### Purification of the "dibromorunning" peptide

A fraction of *Conus textile* venom which caused hyperactivity in mice was purified as described under Methods. The peptide induced rapid running in mice upon intracranial injection. The purification of the peptide is shown in Fig. 1.

**Table 1.** Sequence analysis of tx7a.

| Cycle | Residue | Yield (pmol) | Residue Predicted by Clone |
|---|---|---|---|
| 1 | G | 77.9 | G |
| 2 | M | 94.4 | M |
| 3 | X | – | W |
| 4 | G | 53.1 | G |
| 5 | X | – | E |
| 6 | C | 48.0 | C |
| 7 | K | 25.1 | K |
| 8 | D | 33.4 | D |
| 9 | G | 39.0 | G |
| 10 | L | 43.7 | L |
| 11 | T | 22.5 | T |
| 12 | T | 27.5 | T |
| 13 | C | 29.0 | C |
| 14 | L | 29.9 | L |
| 15 | A | 22.1 | A |
| 16 | O | 34.0 | P |
| 17 | S | 10.3 | S |
| 18 | X | – | E |
| 19 | C | 19.0 | C |
| 20 | C | 26.0 | C |
| 21 | S | 8.6 | S |
| 22 | X | – | E |
| 23 | D | 9.5 | D |
| 24 | C | 12.3 | C |
| 25 | X | – | E |
| 26 | G | 14.8 | G |
| 27 | S | 5.3 | S |
| 28 | C | 8.2 | C |
| 29 | T | 4.4 | T |
| 30 | M | 4.1 | M |
| 31 | X | – | W |

– yield not determined.

Chemical sequence analysis of the peptide was carried out for 32 Edman cycles. As shown in Table I, the sequence Gly-Met-Xxx-Gly-Xxx-Cys-Lys-Asp-

Gly-Leu-Thr-Thr-Cys-Leu-Ala-Hyp-Ser-Xxx-Cys-Cys-Ser-Xxx-Asp-Cys-Xxx-Gly-Ser-Cys-Thr-Met could be assigned up to the 30th cycle. In cycles 3, 5, 18, 22 and 25 (indicated by Xxx) no standard amino acid was obtained at the expected levels. However, in positions 5, 18, 22 and 25, a small amount of glutamate was detected, which we have noticed is a 'tell-tale' sign that is characteristic of the presence of a γ-carboxyglutamate residue. This suggested that four of the unassigned cycles were in fact γ-carboxyglutamate residues.

In order to identify the residue at position 3, the peptide was treated with AspN and the N-terminal heptapeptide fragment analyzed with electrospray ionization (ESI) mass spectrometry (MS). In the positive ESI mass spectrum, intense doublets were observed at m/z 1037.1 / 1039.1 and 1053.1 / 1055.1 (Fig. 2), while in the negative ESI mass spectrum, intense doublets were observed at m/z 1035.1 / 1037.1 (Fig. 2 inset) and 1051.1 / 1053.1 (data not shown). The observed mass in the positive ionization mode (m/z 1037.1) was consistent with the calculated monoisotpic mass (1037.29 Da) for the $[M+H]^+$ of the peptide H-Gly-Met-Trp-Gly-Gla-Cys*-Lys-OH, where Trp is 6-bromotryptophan and Cys* is pyridylethylcysteine. Furthermore, the mass spectra of the peptide had the characteristic doublet suggestive of the presence of bromine, indicating that there might be a 6-bromotryptophan residue in the N-terminal fragment. Thus, the observed mass from the N-terminal fragment peptide fits with the assumption that the absence of PTHs at cycle positions 3 and 5 was due to the presence of 6-bromotryptophan and γ-carboxy-glutamate, respectively. The second set of doublets observed in both the positive (Fig. 2) and negative ionization mode are attributed to oxidation of the methionine residue to methionine sulfoxide. This evidence strongly suggests that within the first thirty amino acids of the sequence,

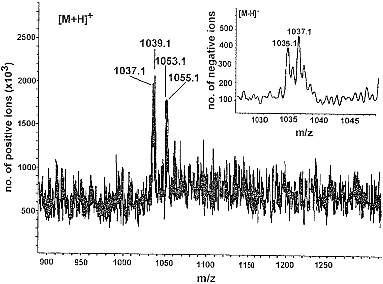

**Figure 2.** ESI mass spectrum, measured in the positive mode, of the N-terminal heptapeptide fragment of tx7a obtained by AspN-treatment. Inset shows the expanded region of the negative ESI mass spectrum.

the five unassigned residues were 6-bromotryptophan at position 3, and γ-carboxyglutamates at positions 5, 18, 22 and 25.

The ESI mass spectrum of the intact peptide contained fragments at m/z 1226.4, 1231.7, 1244.2, 1249.3, 1261.8, 1266.8, 1279.7 and 1284.9 (positive ionization mode) which we interpret as $[M+3H]^{3+}$, $[M_{ox}+3H]^{3+}$, $[M+Fe]^{3+}$, $[M_{ox}+Fe]^{3+}$, $[M+Fe-3H]^{3+}$, $[M_{ox}+Fe-3H]^{3+}$, $[M+2Fe-6H]^{3+}$ and $[M_{ox}+2Fe-6H]^{3+}$ (where $M_{ox}$ indicates the methionine sulfoxide species; Fig. 3). The inset in Fig. 3 shows the resolved isotope distribution superimposed on the normal spectrum, the spacing between the isotopomers in each of the different species indicated the charge state (+3). In the negative ionization mode the spectrum contains intense species at m/z 1224.2, 1227.8, 1242.2, 1247.5, 1259.7, 1265.3, 1227.6 and 1282.6 which we interpret as $[M-3H]^{3-}$, $[M_{ox}-3H]^{3-}$, $[M+Fe-6H]^{3-}$, $[M_{ox}+Fe-6H]^{3-}$, $[M+2Fe-9H]^{3-}$, $[M_{ox}+2Fe-9H]^{3-}$, $[M+3Fe-12H]^{3-}$ and $[M_{ox}+3Fe-12H]^{3}$. A close correspondence is observed between the species observed in the positive and negative ESI spectra (Fig. 4A and 4B). A mass difference of 6 Da (m/z 2 for triply-charged ions) is consistent with a difference of six protons between the positive and negative species, which supports the interpretation given above. The MS/MS spectrum measured in the negative ionization mode of the m/z 1242.2 species contained intense fragment ions at m/z 1182.7, 1197.2, 1211.8 and 1226.3 corresponding with the loss of one to four molecules of $CO_2$ (consistent with the presence of four γ-carboxyglutamate residues), but no other fragment ions (spectrum not shown).

However, the observed average mass (M) of the intact peptide of 3676.2 Da was greater than the mass predicted for the 30 AA sequence obtained by the Edman analysis (3412.45 Da). The mass difference (»264 Da) between the observed ESIMS and the calculated mass for the thirty residues (including the Trp at position 3 and four Gla residues) was consistent with an additional residue of 6-bromotryptophan. This result suggested an additional Trp residue at the C-

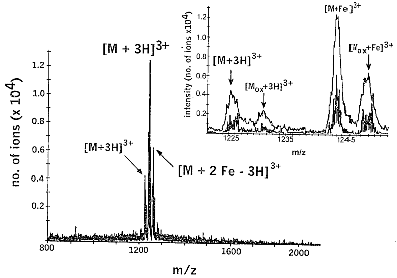

**Figure 3.** ESI mass spectrum, measured in the positive mode, of intact tx7a. Inset shows an expanded range measured in both normal and maximum-resolution settings.

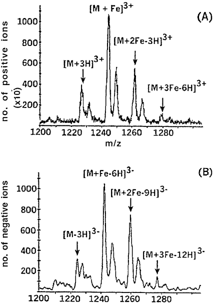

**Figure 4.** ESI mass spectra of intact tx7a measured in both the positive (A) and negative (B) ionization modes. The close correspondence between the species observed in A and B and the mass shift of 6 Da was used to assign the species.

terminal end (which would not have yielded an assignable residue under the conditions of Edman analysis utilized).

We have previously determined that the γ-carboxyglutamate-containing conotoxin tx5a can retain a single ferric cation and lose five protons to form a negatively-charged species (Walker et al. 1999; A.G. Craig, J.P. Spier, S. Rosamila, V. Fursey, M.B. Lirazan, submitted to J. Amer. Soc. Mass Spectrom, in press). The close correspondence between the positive and negative ESI spectra and the mass difference of 6 Da between corresponding positive and negative species (m/z 2 for triply-charged ions) listed above strongly supports the assignment given and therefore the retention of three ferric cations by the molecule and the loss of up to 12 protons to form negatively charged species. Since only six acidic residues are present in this peptide (4 γ-carboxyglutamates and 2 aspartates) which could give up a maximum of 10 protons, protons must be removed from two of the six hydroxy amino acids present in the peptide. The MS/MS spectrum in which four molecules of $CO_2$ are lost without loss of a ferric cation and the absence of other high mass fragment ions in the MS/MS spectrum are consistent with our previous findings for the ferric-cationized conotoxin tx5a.

### Cloning

A cDNA clone, Tx7.1, encoding the peptide was obtained using PCR as described under Methods; the amino acid sequence predicted by the cDNA clone is shown in Table I. Consistent with the postulate that there was a 6-bromotryptophan residue at position 3, a Trp codon was found. The sequence deduced from the clone was also consistent with γ-carboxyglutamate at positions 5, 18, 22 and 25, since Glu codons were found at these positions. Most importantly, the clone did predict a C-terminal tryptophan codon, consistent with 6-bromotryptophan as the C-terminal amino acid.

These results lead to the following sequence assignment for the peptide:

GMWGγCKDGLTTCLAOSγCCSγDCγGSCTMW
(where W = Trp and γ = Gla)

This is the first peptide known to contain more than one Trp residue. Injection of six mice at doses between 50 to 80 pmol/g of the purified peptide elicited continuous running for more than an hour in all of the animals. We refer to the peptide as the "dibromorunning peptide." In conformation with the nomenclature proposed by McIntosh et al. (McIntosh et al. 1999), the peptide is given the provisional technical designation tx7a, encoded by the cDNA clone Tx7.1.

## Discussion

We characterized a 31-amino acid peptide with three disulfide bonds that causes a characteristic rapid-running hyperactivity when injected i.c. into mice. The presence of two residues of 6-bromotryptophan, and four residues of γ-carboxyglutamate in the peptide is especially noteworthy. Together, the cysteines involved in disulfide bonding, the γ-carboxyglutamate residues and the 6-bromotryptophan residues account for 12 of the 31 amino acids in the peptide. Like the bromosleeper peptide, it has a disulfide pattern that superficially, at least, resembles that of the O-superfamily. However, additional cloning data (R. Shetty and C. Walker, unpublished results) indicate that this peptide is not a member of the O-superfamily, since its signal sequence differs from that of other O-superfamily members (even though the arrangement of cysteines appears to be homologous to the O-superfamily). Further characterization of the peptide and an identification of the role of γ-carboxyglutamate and 6-bromotryptophan residues awaits chemical synthesis and structure/function studies.

The finding that two unusual post-translational modifications, bromination of tryptophan and γ-carboxylation of glutamate residues occur together in three unrelated *Conus* peptides strongly suggests that the combination of these two post-translational modifications yields a functional motif important for the neuropharmacological activity of these peptides. As is typical of *Conus* peptides, their structures and symptoms elicited are very diverse. Thus, although the present peptide, tx7a, has a cysteine framework similar to that of the bromosleeper peptide previously characterized (rather than to that of conotoxin tx5a from the same venom, *Conus textile*), this peptide makes mice hyperactive when injected intracranially while as previously reported, the bromosleeper peptide induces a sleep-like state. Although both tx7a and tx5a elicit excitatory symptomatology, they appear to be completely unrelated structurally. Thus, the subset of conotoxins which have both 6-bromotryptophan and γ-carboxyglutamate are a diverse, highly potent and intriguing group of *Conus* venom peptides.

The only conopeptide with 6-bromotryptophan whose function is known is σ-conotoxin from *Conus geographus* which targets the $5HT_3$ receptor; it was postulated that the single residue of Trp might interact with the serotonin (5HT) binding site (England et al. 1998). In principle, one rationale for bromotryptophan in conopeptides is that all Trp-containing peptides could be targeted to 5HT binding sites on the diverse types of serotonin receptors. The discovery of a peptide with two Trp residues makes this explanation less likely, and suggests that the brominated residues have other mechanistic roles.

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# Electrochemically Synthesized Polymer-based pH Sensors

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A polymer-coated wire electrode was fabricated by galvanostatic electrochemical polymerization of monomer and this was evaluated for pH response. Four different monomers were investigated namely, pyrrole (Py), 3-(2,5-dihydroxybenzyl)pyrrole (PyQH), aniline (An) and *o*-phenylenediamine (oPD). The polymers were synthesized with perchlorate or bovine serum albumin (BSA) dopants in a propylene carbonate or universal buffer electrolytes, respectively. The potentiometric characteristics of the resulting films show sub- to nearly Nernstian responses. The range of electrode sensitivities (25° C) for the four polymers used are –43.2 mV/pH for PPy, -46.0 mV/pH for PPyQH, -42.1 mV/pH for PAn and –50.7 mV/pH for PoPD. All has linear working range of pH 3 to 10 and average response time of 3 to 5 min. Electrode stability was maintained without the need of pre-treatment prior to use or immersion in solution when not in use. X-ray photoelectron spectroscopy (XPS) was used to probe the composition and surface characteristics of the electrodes. Conducting polymers are ideally suited for sensor applications because they not only exhibit high conductivity and electroactivity but they could also be used as general matrix and further modified with other compounds in order to change selectivity. The conducting polymers studied offer potential for pH sensor devices and could find wide applications.

Key words: *conducting polymers, polypyrrole, polyaniline, poly (o-phenylenediamine), pH electrode, polymer-modified sensor*

The past decade had shown a remarkable outpouring of research activity in the field of chemically modified electrodes. Chemically modified electrodes have been employed as potentiometric sensors. The most common method for electrode modification involved covalent linking of suitable groups to electrode surface. In addition to covalent attachment, a more practical approach is coating the electrode surface of polymeric films (Aquino-Binag et al. 1994). These polymeric films have been formed by casting the film on electrode surface or electrodeposition (Heineman et al 1980, Aquino-Binag et al. 1996).

Polymerization of conducting polymers onto electrode surface could be done electrochemically. There has been a lot of interest in conducting polymers for their potential in the development of chemical sensor technology (Street 1983, Walls 1989). Particularly interesting are the polypyrrole-, polyaniline- and poly(*o*-phenylenediamine)- based pH sensors. Although the conventional glass electrode proved to be convenient and precise potentiometric pH indicator, it has its inherent limitations. It is not applicable in cases where miniaturization, compactness and durability are desired. On the other hand, the use of electrochemically synthesized conducting polymer membranes in pH-sensing devices presents the advantages of miniaturization, simple fabrication and robustness.

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This study demonstrates the analytical utility of conducting polymer-based pH sensors as attractive alternatives to the conventional pH glass electrode. In addition, this study is the first to describe the surface and potentiometric properties of the four different conducting polymers synthesized electrochemically.

## Methods

### Materials and Electrode Fabrication

All solutions were prepared using analytical grade reagents and distilled water. Pyrrole (Sigma) and aniline (Sigma) monomers were distilled before use. *o*-Phenylenediamine (Sigma) monomer was doubly crystallized in dichloromethane (Sigma) and decolorized with activated carbon during the first crystallization prior to use. While 3-(2,5-dihydroxybenzyl)pyrrole (PyQH) was synthesized and the synthetic aspects of this methodology have been reported previously in more detail (Aquino-Binag et al 1995).

The pH sensor was prepared by electropolymerization of monomer onto a platinum wire (0.26 cm$^2$) under constant current against a carbon rod (140 cm$^2$). The platinum wire was polished with alumina paste, sonicated and rinsed with distilled water prior to use. The anode (Pt) and the cathode (carbon rod) were faced parallel separated by a distance of 1.0 cm. Current was passed through a current limiting device, which was placed in series with the voltage source and the cell (Tongol et al. 1999). The current limiting device controls the current (2.5 mA) through the cell. For Py, An and oPD monomers, the polymerization solution consisted of 0.1 monomer, 2.5 mg BSA (Merck) dopant and phosphate buffer system (pH 7.0). While for the PyQH, the polymerization solution consisted of 0.1 M monomer, 0.1 M perchlorate dopant in propylene carbonate electrolyte solution with 1% water.

### Electrode Performance Characteristics

Potentiometric and pH measurements were made with a pH/mV meter (Activon Model 209) and a calomel reference electrode (Metrohm Model B-NS 14/15) or Ag/AgCl reference electrode (Orion 900200). Potentiometric response curves were measured using the following cell:

Pt / conducting polymer / sample // SCE or Ag/AgCl

Calibration plots were obtained for solutions containing hydrogen ion concentrations ranging from $10^{-3}$ to $10^{-10}$ M. The pH sensor and the SCE or Ag/AgCl electrode were connected to the voltmeter and then both electrodes were immersed in a beaker containing the test solution. The output voltage of the sensor was monitored against SCE or Ag/AgCl reference electrode using a voltmeter (Metrohm). Data acquisition was done by interfacing the measurement set-up to a computer through an 8-channel, 12-bit ADC card (Innovatronix Inc). The data acquisition set-up enabled monitoring, storage and retrieval of experimental data. All measurements were made at room temperature (25° C ± 2) with the solution constantly stirred.

The test solutions consisted of 0.04 M Britton-Robinson universal buffer whose pH values were adjusted by the addition of 3 M NaOH to the appropriate values. The response time of the polymer indicator electrode was evaluated by immersion in increasing concentrations of $H^+$ solutions. The sensor signal at a particular pH was monitored until a steady state value was reached and this steady state response was taken as the sensor signal corresponding to a particular pH.

### Electrode Surface Characterization

X-ray photoelectron spectroscopy (XPS) was used to analyze the polymer films grown on Pt wire electrodes. Experiments were performed using a Kratos AXIS surface analysis system. A Mg anode x-ray source was operated at 190 W. The maximum energy resolution of the concentric hemispherical analyzer (CHA) was 0.87 eV operated for XPS analysis in the fixed analyzer transmission (FAT) mode with a pass energy of 20 eV for Ag $3d_{5/2}$ emission (Walls 1989). The electron binding energies ($E_B$) were calibrated relative to a saturated hydrocarbon C *1s* component peak at $E_B$ = 285.0 eV (Beamson & Briggs 1992). Peak areas were quantified using appropriate sensitivity factors.

SEM studies of selected polymer samples (PPy, PAn, PPyQH) were performed using a JEOL JXA-840 scanning microanalyzer. The microscope chamber was maintained at a pressure below $<10^{-6}$ Torr. The SEM filament voltage was operated at 3.0 kV and a current of 6 nA. The surface morphology of poly(*o*-phenylenediamine) polymer film was studied using a JEOL JSM-35C SEM. An accelerating voltage of 25 kV was used limiting the load current to within 100 mA.

## Results and Discussion

### Sensor Performance

The chemical structures of the four different monomers namely, pyrrole (Py), 3-(2,5-dihydroxy-benzyl)pyrrole (PyQH), aniline (An) and *o*-phenylenediamine (oPD) are shown in Figure 1. All have the characteristic conjugated structures with alternating single, double bonds along the polymer chains. Among the four polymers synthesized, the PoPD-coated sensor gave the best sensitivity with a near-Nernstian response of -50.7 mV/pH as shown in Figure 2. The response of electrodes coated with parent PPy, PPyQH and PAn are all sub-Nernstian responses with slopes of –43.2, -46.0 and –42.1 mV/pH, respectively.

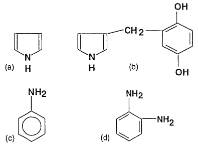

Figure 1. The four monomers used as matrices for $H^+$ sensing are (a) pyrrole (Py), (b) 3-(2,5-dihydroxybenzyl)pyrrole (PyQH), (c) aniline (An) and (d) *o*-phenylenediamine (oPD).

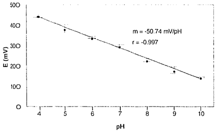

Figure 2. The potentiometric response of PoPD/BSA-coated Pt wires as a plot of potential (mV) against pH, based on the mean of three (3) trials. The error bars represent the deviation of each trial from the mean.

*Polypyrrole (PPy).* The pH sensitivity of polypyrrole has been attributed to the reversible protonation and deprotonation of the nitrogen atom of PPy as the solution pH changes (Inganas et al 1984). This parent PPy polymer is a uniform, well adhering black membrane film with conductivity range of $10^0$ - $10^4$ $(\Omega cm)^{-1}$. The PPy/BSA sensor has good sensitivity with sub-Nernstian slope of –43.2 mV/pH, has good correlation coefficient, r = 0.993 and a linear working range from pH 3 to 10. The electrode response time is 3-4 min (Fig. 3) for each of the buffer solutions (pH 3-10). It has high reproducibility (< 3% c.v.), minimum electrode drift (0.1 mV/min), high selectivity ($k_{ij} = 10^{-7}$) and electrode lifetime of 40 days.

*Poly-3-(2,5-dihydroxybenzyl)pyrrole (PPyQH).* The hydroquinone-functionalized PPy has a sub-Nernstian response of –46.0 mV/pH. This has minimal electrode hysteresis, a lower detection limit thus better sensitivity. It has a wide linear working range from pH 3-10, with minimal carryover of ±1.9 mV. A thin, uniform brown polymeric film of PPyQH with conductivity range of $10^0$-$10^2$ $(\Omega cm)^{-1}$ was grown on Pt wire. The increase in the sensitivity to $H^+$ relative to the parent PPy is due to the

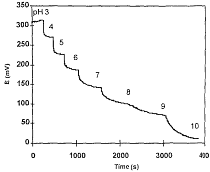

Figure 3. Typical response of the PPy/BSA pH sensor. Polymerization condition: 0.1 M pyrrole, 2.9 mg BSA, 0.1 M phosphate buffer (pH 7.0); 10 mA/cm$^2$; 3 min.

additional pH sensitive quinhydrone functionality covalently bound to the PPy backbone.

*Polyaniline (PAn).* The resulting PAn was an emerald green film with conductivity of $10^{-5}$ $(\Omega cm)^{-1}$. The pH sensitivity of PAn has been attributed to the reversible protonation and deprotonation of the nitrogen of PAn as the solution pH changes. The electrode has a sub-Nernstian slope of –42.1 mV/pH with high linearity (r = 0.9985). It has stable response with minimal electrode drift (RSD < 3%), minimal hysteresis ($\Delta m$ = 5.83 mV/pH) and high reproducibility (RSD ~4%) and an electrode lifetime of more than 1.5 months. Figure 4 shows a typical electrode drift study of PAn/BSA pH sensor. The electrode showed a minimal drift at pH 7 with an RSD of 1.7% and RSD of 2.3% at pH 4, signifying its appropriateness in neutral and acidic media. Due to the instability of polyaniline in basic solutions, there has been a great increase in the RSD at pH 8.

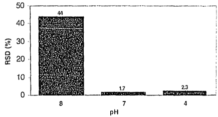

Figure 4. Electrode drift study of PAn/BSA. Performance of the electrode in acidic (pH 4; RSD = 2.3%), neutral (pH 7; RSD = 1.7 %) and alkaline pH (pH 8; RSD = 44%).

*Poly(o-phenylenediamine) (PoPD).* The pH sensitivity of PoPD has been attributed to the protonation and deprotonation of amine linkages in the polymer (Heineman et al 1980) and to permselectivity of PoPD to hydrogen ions (Cheek et al 1983, Ohsaka et al 1987, Sasso et al 1990). The latter enhances the potentiometric response of the sensor because of its high selectivity to hydrogen ions. This brown uniform polymer coated electrode has conductivity range of $10^{-1}$-$10^{-4}$ $(\Omega cm)^{-1}$. The sensor has a near Nernstian response of –50.7 mV/pH, high linearity of r = 0.997 and response time of 7 min (Tongol et al 1999). It has high repeatability (RSD ~ 6 %), high reproducibility (RSD < 3%), low electrode drift (RSD < 2%), low hysteresis ($\Delta m$ = 1.06 mV/pH) and high selectivity ($k_{ij}$ = $10^{-6}$ to $10^{-8}$). Figure 5 shows a typical reproducibility study of the PoPD-based pH electrode.

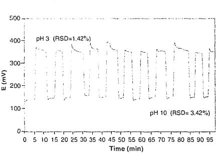

Figure 5. Plot of potential (mV) against pH at eleven replicate and alternate measurements at pH 3 and pH 10 of PoPD/BSA electrode.

## Surface Studies

*X-ray Photoelectron Spectroscopic Analysis.* The information on the types of atoms and the chemical bonding of an element at or near to the surface are determined by XPS wide scans as well as the high resolution scans of each element. Figure 6 shows a typical wide scan spectrum of PoPD/BSA film sensor. The characteristic N *1s* component peak is present in all the polymers investigated centering at binding energy of ~400 eV. The BSA dopant with a structure consisting of carbohydrate-free peptide chain connecting

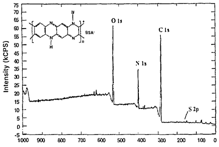

**Figure 6.** XPS wide scan spectrum (0-1000 eV) showing the major components of PoPD/BSA film membrane: C *1s* (~285.0 eV), O *1s* (~531.0 eV), N *1s* (~400.0 eV) and S *2p* (~165.0 eV).

four globular segments of unequal size, stabilized with seventeen S-S bridges has the doublet S *2p* component peak centered at ~168.5 eV and ~163.7 eV. For polymers using perchlorate ($ClO_4^-$) as dopant, there was the presence of a doublet Cl *2p* component peak centered at ~532.5 eV and ~535 eV. XPS is a powerful surface analytical tool to investigate the elemental compositions and chemical environment of elements on the topmost polymeric layers.

*Scanning Electron Microscopic Analysis.* SEM studies of surface morphology show that the parent PPy has non-uniform rough surface in contrast with the smooth surface of the hydroquinone-functionalized PPy. A series of SEM micrographs of the parent PPy and PPyQH shows that structure changes with the type of functionality attached to the PPy backbone for the same polymerization conditions. The parent PPy/$ClO_4^-$ has a cauliflower type surface, while the PPyQH/$ClO_4^-$ is smooth layered film (Aquino-Binag et al. 1996).

PPy with other dopants show rougher surface like the PPy/BSA membrane is relatively more sensitive (-43.2 mV/pH) than the *p*-toluene sulfonate doped polypyrrole (PPy/$pTSO_4^{2-}$) film (-35.4 mV/pH) reported in the literature (Aquino-Binag 1996). SEM micrograph of the PPy/BSA film showed a fern-like morphology, in contrast to the more uniform cauliflower-like morphology of the PPy/$pTSO_4^{2-}$ film. PAn/BSA has rugged non-uniform surface, while PoPD/BSA has fluffy surface with circular nodules.

## Conclusions

The electrochemical properties and surface characteristics of four membrane films grown on Pt wires showed great potentials as attractive alternatives to the conventional pH glass electrode. The advantages of electrode surface modification with conducting polymers in terms of processability are ease of sensor fabrication, mechanical stability and enhanced response reproducibility. Surface-sensitive techniques like XPS has shown the chemical and physical nature of topmost layers of membrane coatings of the electrodes.

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**RESEARCH**

# Effect of Cooking Method on Amylopectin Staling as Monitored by Instron Hardness[†]

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Among high-amylose rices, glycemic index, an index of amylose staling of cooked rice, is affected by starch gelatinization temperature (GT) when cooked in optimum water level but not when cooked in excess water to minimum cooking time. Regardless of amylose content (AC), low GT rice cooked by rice cooker showed less amylopectin staling than cooked intermediate GT rice. Hence, we studied the effect of cooking by excess water method on amylopectin staling of cooked rice on three high AC rices, PSB Rc 6 (low GT) and PSB Rc 2 and PSB Rc 56 (intermediate GT), two low-GT low-AC rices, IR24 and Sinandomeng, and two waxy rices, Malagkit Sungsong (low GT) and Tapol (high GT). The results suggest that although cooking by excess water method reduced Instron hardness values, varietal differences in amylopectin staling were still evident and in the same ranking as that established by the rice cooker method. Varietal differences in staled amylopectin melting endotherm in rice flour cooked in excess water were verified by differential scanning calorimetry.

**Key words**: *Slow starch (amylopectin) retrogradation, differential scanning calorimetry, amylose staling, glycemic index, resistant starch*

In the rice breeding program, eating quality of freshly cooked and cold cooked rice are important properties to monitor in promising entries. Hardness of freshly cooked rice is mainly due to amylose staling, whereas further hardening of cooked rice during storage is due mainly to amylopectin staling. In addition, staling should contribute to spoilage because of the free water released when starch molecules retrograde in starch gels.

Amylose staling of cooked rice as indexed by insulin and glycemic response decreases with increasing amylose content (AC) regardless of cooking method (Goddard et al 1984, Juliano & Goddard 1986, Juliano et al 1989, Miller et al 1992, Larsen et al 1996, Tetens et al 1997). Among high-AC Philippine rices (>25%), milled rices cooked on a stove for 22 min with identical optimum water-rice ratio (2.5–2.7, 68–69% water) showed higher glycemic index for one low-gelatinization temperature (GT ≥ 70°C) rice (91–94%) than two intermediate–GT (> 70°C) rices (61–72%) (Panlasigui 1989, Panlasigui et al 1991). When cooked in excess water to minimum cooking time (14–20 min, 69–70% water), the three rices showed similar but low glycemic index (75–81%). Total and fermentable resistant starch in rats, another index of amylose staling, was likewise lower in low-GT and low–AC rices cooked by the rice cooker than higher GT and higher AC rices (Eggum et al 1993).

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Regardless of AC type, low-GT rice cooked by rice cooker showed less amylopectin staling (as indexed by Instron hardness measured with a 10-cm$^2$ OTMS cell) than cooked intermediate-GT rice (Perez et al 1993, Villareal et al 1993, 1997). The greatest amylopectin staling was observed for high-GT waxy rice, such as Tapol (Villareal et al 1993). Even among low-GT, low-AC rices, Sinandomeng had less amylopectin staling of cooked rice than IR24 (Roferos & Juliano 1997). Amylopectin staling of nonwaxy rice starch gels indexed by storage modulus G' in oscillating viscometry and staled amylopectin melting enthalpy at 45-60°C by differential scanning calorimetry (DSC) correlated with AC and was higher in starches with higher GT (Perez et al 1993, Biliaderis & Juliano 1993).

Since amylose staling is affected by cooking method, we determined whether or not the differences in amylopectin staling shown in the rice cooker method are also minimized by cooking in excess water to minimum and optimum cooking time.

## Materials and Methods

Rough rices were obtained from the PhilRice yield trials. They included three high-AC rices from the 1997 wet-season crop, low-GT PSB Rc 6 and intermediate-GT PSB Rc 2 and PSB Rc 56; two 1997 dry-season, low-GT, low-AC rices, IR24 and Sinandomeng; and waxy rices, low-GT Malagkit Sungsong from the 1997 wet-season crop and 1994 market samples of purple high-GT Tapol brown rice from Bacolod City (stored in a freezer upon receipt).

Rough rice aged at least 4 mo from harvest were dehulled with a Satake THU 35 type dehusker and the brown rice was milled in a McGill miller no. 2. Head rice was obtained for nonwaxy rices using a Satake testing rice grader TRG 05A with suitable indented cylinder to remove brokens (< 3/4 whole-grain length). Samples were analyzed for apparent AC by iodine colorimetry (Juliano et al 1981), GT by alkali spreading value in 1.7% KOH (Little et al 1958), and moisture content from loss of weight in a 130°C forced-draft oven for 1 h (AACC 1982).

Cooking was done in duplicate, four 150-mL beakers at a time, in a Toshiba RC-4B rice cooker (400 w) with 200 mL water in the outer pot (Perez et al 1993). Milled rice (20 g) was placed in each beaker and distilled water (24 mL) was added. The cooker was turned on and 20-min boiling time was recorded, after which the cooker was turned off and let stand for an additional 10 min. Samples were weighed to calculate water-uptake ratio and cooled in a plastic bag for 1 h prior to determination in duplicate of Instron hardness using a 10-cm$^2$ Ottawa Texture Measuring System cell (Perez et al 1993). Cooked rice was staled by at least 4 h at 2-4°C, followed by at least 4 h at 42°C (Slade et al 1987) before Instron hardness measurement.

Minimum cooking time was measured by the method of Ranghino (1966). Milled rice in a sieve basket was cooked in 6 times the amount of boiling water for its mimimum cooking time and its optimum cooking time (minimum cooking time + 2 min). Weight of cooked rice was recorded for calculation of water uptake ratio. Amount of dissolved solids was measured on an aliquot of the cooking liquor to determine leached solids. Samples were cooled for 1 h in a plastic bag before measurement of Instron hardness.

Differential scanning calorimetry (DSC) utilized a Shimadzu DSC-50 unit with hermetically sealed, high-pressure (50-bar) Shimadzu pan (Biliaderis & Juliano 1993). Duplicate rice flour samples (100-mesh, 4 mg) were sealed with water corresponding to the water uptake ratio of 1.4-2.3 and cooked at the rate of 10°C/min up to 130°C. The calorimeter pans were then cooled and subjected to amylopectin staling (4 h at 2-4°C and 4 h at 42°C or 3-5 days in the refrigerator) before measurement of staled amylopectin endotherm at 45-75°C at the rate of 10°C/min.

Instron and DSC enthalpy data were subjected to analysis of variance and Duncan's (1955) multiple range test.

## Results and Discussion

**Optimum water method.** The low-GT, high-AC PSB Rc 6 had harder freshly cooked rice than the two other high-AC rices with intermediate GT, PSB Rc 2 and PSB Rc 56 (Table 1). As expected (Perez et al 1993), the three samples had similar staled cooked rice hardness values. The two low-GT, low-AC rices had similar freshly cooked rice hardness, and IR24 became harder on amylopectin staling. The waxy rices showed softest freshly cooked rice, but the high-GT Tapol became as hard as high-AC staled rice on staling. Water content of the samples at the same water-rice ratio during cooking was 57-58%, to simulate the PhilRice cooking method with water-rice ratio of 1.5, resulting in a water-uptake ratio of 1.1-1.2 (58-60% water). Similar results were obtained by Roferos & Juliano (1997) using a similar lower water level and by Perez et al (1993) using a water-rice ratio of 1.7-2.1 for nonwaxy rice and Villareal et al (1993) using water-rice ratio of 1.3 for waxy rice.

**Cooking in excess water.** Higher apparent water content was obtained with cooking in excess water to minimum cooking time (Table 2). Water content

**Table 1.** Instron hardness of freshly cooked and amylopectin-staled cooked milled rices of similar amylose contents (AC) cooked at optimum water content[a].

| Variety or line name | Crop[b] | AC[c] type | GT[d] type | Instron hardness[e] (kg/cm$^2$) | |
|---|---|---|---|---|---|
| | | | | Freshly cooked | Staled cooked |
| PSB Rc 6 | 97WS | High | Low | 3.3 ± 0.1e | 3.8 ± 0.3de |
| PSB Rc 2 | 97WS | High | Interm. | 3.0 ± 0.0d | 3.8 ± 0.1e |
| PSB Rc 56 | 97WS | High | Interm. | 2.8 ± 0.1d | 3.8 ± 0.0de |
| Sinandomeng | 97DS | Low | Low | 1.4 ± 0.2c | 1.4 ± 0.2b |
| IR24 | 97DS | Low | Low | 1.0 ± 0.0ab | 2.4 ± 0.1c |
| Malagkit Sungsong | 97WS | Waxy | Low | 0.8 ± 0.0a | 1.0 ± 0.1a |
| Tapol | 94 | Waxy | High | 1.2 ± 0.0bc | 3.5 ± 0.3d |

[a] Water-rice ratio 1.2, 57-58% water in cooked rice.
[b] WS = wet season, DS = dry season; [c] AC = apparent amylose content; [d] GT = final gelatinization temperature; [e] Mean ± standard deviation. Means in the same column followed by the same letter are not significantly different at P = 0.05 by Duncan's (1955) multiple range test. LSD (0.05) for two fresh·staled means = 0.3.

**Table 2.** Instron hardness of freshly cooked and amylopectin-staled cooked milled rices of similar amylose contents cooked in excess water at minimum cooking time[a].

| Variety or line | AC type | GT type | Minimum cooking time (min) | Water content (%)[a] | Instron hardness[b] (kg/cm$^2$) | |
|---|---|---|---|---|---|---|
| | | | | | Freshly cooked | Staled cooked |
| PSB Rc 6 | High | Low | 15 | 70-72 | 0.73 ± 0.01e | 0.89 ± 0.04f |
| PSB Rc 2 | High | Interm. | 18 | 71-72 | 0.57 ± 0.01c | 0.83 ± 0.01e |
| PSB Rc 56 | High | Interm. | 18 | 72-73 | 0.64 ± 0.06d | 0.88 ± 0.01ef |
| Sinandomeng | Low | Low | 15 | 68-70 | 0.52 ± 0.01c | 0.56 ± 0.03b |
| IR24 | Low | Low | 16 | 67-70 | 0.36 ± 0.01a | 0.67 ± 0.01c |
| Malagkit Sungsong | Waxy | Low | 16 | 64 | 0.44 ± 0.00b | 0.45 ± 0.01a |
| Tapol | Waxy | High | 23 | 69 | 0.55 ± 0.00c | 0.76 ± 0.06d |

[a] Corrected for leached-out solids during boiling; [b] Mean ± standard deviation. Means in the same column followed by the same letter are not significantly different at P = 0.05 by Duncan's (1955) multiple range test. LSD (0.05) for two fresh·staled means = 0.05.

tended to decrease with decreasing AC, probably due to greater starch leaching in the lower AC grains. Minimum cooking time ranged from 15-23 min. PSB Rc 6 had the hardest freshly cooked grain, but the staled grain showed more staling for PSB Rc 2 and PSB Rc 56 than in PSB Rc 6, resulting in similar staled grain hardness. The low-AC rices had similar freshly cooked and staled grain hardness values. As to the two waxy rices, Tapol cooked rice was harder than Malagkit Sungsong in both freshly cooked and staled cooked forms. Tapol staled cooked rice was even harder than low-AC staled cooked rices, but softer than high-AC staled cooked rices.

Cooking in excess water for an additional 2 min or to optimum cooking time increased water content to 77-80% and resulted in softer cooked rice (Table 3). The high-AC rices still showed amylopectin staling, but the low-AC rices did not. Dissolved solids in cooking water was 7-10% of milled rice weight.

The calorimetry data on milled-rice flours confirmed the higher staled amylopectin melting endotherm for PSB Rc 2 and PSB Rc 56 over PSB Rc 6 (Table 4), in conformity with Instron hardness data. The calorimetry data for the low-AC samples Sinandomeng and IR24 were not conclusive in contrast to the Instron hardness data in Table 1 and 2, but the data for waxy rices showed higher endotherm for high-GT Tapol. The higher peak temperatures (>60°C) obtained may be due to the use of 100-mesh flours in the study, rather than rice starch granules. Normand & Marshall (1989) reported higher peak temperatures for flours than for starch granules.

**Table 3.** Instron hardness of freshly cooked and amylopectin-staled cooked milled rices of similar amylose contents cooked in excess water at optimum cooking time[a].

| Variety or line | AC type | GT type | Optimum cooking time (min) | Water content (%) | Instron hardness[b] (kg/cm²) | |
|---|---|---|---|---|---|---|
| | | | | | Freshly cooked | Staled cooked |
| PSB Rc 6 | High | Low | 17 | 75 | 0.68 ± 0.03c | 0.74 ± 0.03c |
| PSB Rc 2 | High | Interm. | 20 | 74 | 0.56 ± 0.00b | 0.77 ± 0.00c |
| PSB Rc 56 | High | Interm. | 20 | 74 | 0.72 ± 0.03c | 0.86 ± 0.00d |
| Sinandomeng | Low | Low | 17 | 74 | 0.46 ± 0.03a | 0.40 ± 0.00b |
| IR24 | Low | Low | 18 | 77 | 0.44 ± 0.00a | 0.34 ± 0.00a |

[a] Corrected for leached-out solids during boiling.

[b] Mean ± standard deviation. Means in the same column followed by the same letter are not significantly different at P = 0.05 by Duncan's (1955) multiple range test. LSD (0.05) for two fresh·staled means = 0.04.

The results indicate that regardless of cooking method, amylopectin staling of high-AC and waxy rices were still evident based on the increase in Instron hardness and were greater for intermediate- and high-GT rices than in low-GT rices as reported by Perez et al (1993) and Villareal et al (1993). This was confirmed by differential scanning calorimetry at water-rice ratio 1.4-2.3.

Perdon & Juliano (1975) reported that high-GT waxy rice starch had higher sedimentation coefficient in dimethylsulfoxide and gel viscosity of 6% starch in 0.2M KOH with a Wells Brookfield cone-plate viscometer. Neutral gel consistency of 10% milled rice flour in 0.15M potassium acetate was harder (lower length) in high-GT waxy rice than in low-GT waxy rice (Antonio & Juliano 1974, Juliano et al 1974, Antonio

**Table 4.** Differential scanning calorimetry data of cooked milled rice flour in water for staled-amylopectin melting enthalpy after 3-5 d cooling in the refrigerator.

| Variety or line | Water-rice ratio | Staled-amylopectin melting | |
|---|---|---|---|
| | | Enthalpy[a] (J/g) | Peak temp. (°C) |
| PSB Rc 6 | 1.7 - 2.0 | 0.9 ± 0.0a | 67.0 ± 2.9 |
| PSB Rc 2 | 2.0 | 4.3 ± 0.5c | 63.2 ± 0.8 |
| PSB Rc 56 | 2.3 | 3.9 ± 0.3c | 62.4 ± 1.0 |
| Sinandomeng | 1.75 | 1.6 ± 0.8ab | 65.3 ± 3.9 |
| IR24 | 2.0 - 2.2 | 0.9 ± 0.4a | 67.2 ± 8.2 |
| Malagkit Sungsong | 1.4 - 1.43 | 2.0 ± 0.1b | 62.2 ± 0.7 |
| Tapol | 1.7 - 2.0 | 4.7 ± 0.5c | 62.4 ± 0.6 |

[a] Based on 11% moisture and 90% starch in dry matter of milled rice; [b] Mean ± standard deviation. Means in the same column followed by the same letter are not significantly different at P = 0.05 by Duncan's (1955) multiple range test.

et al 1975). However, Villareal et al (1997) reported that high-GT RD4 and Tapol isoamylase-debranched waxy amylopectin had similar mean weight-average degree of polymerization ($DP_w$) and high performance liquid chromatography pattern as low-GT waxy samples, but Tapol had slightly more of the higher $DP_w$ fractions $B_2$ and $B_3$. Unfortunately, number-average degree of polymerization ($DP_n$) of native amylopectin was not measured. Evidently the difference between high-GT waxy amylopectin and low-GT waxy amylopectin is not in molecular structure, but in molecular size.

Amylose leaches out of the starch granule during cooking and stales faster than amylopectin, which remains in the gelatinized granule. Thus in the excess water cooking method, the leached amylose is dispersed in the excess cooking water, but it settles on the cooked rice surface as the water is completely absorbed by the rice grain in the rice cooker method. This difference in amylose concentration in the cooking water may affect the nature of amylose staling. By contrast, amylopectin remains in the gelatinized starch and the increase in water content of the granule in the excess water method is much less than that for amylose. Among high-AC rices, low-GT starch amylopectin has longer chain length than intermediate-GT starch amylopectin, contributing to hard gel consistency and high Amylograph and RVA setback and consistency (Perez et al 1993, Villareal et al 1997). Less soluble amylose leaches out during cooking of low-GT high-AC rice than in intermediate-GT rice (Juliano et al 1987) as the high-iodine-binding amylopectin remains in the residual granule but this amylopectin contributes equally to harder cooked rice texture. Among low-AC rices, mean amylopectin chain lengths were also similar (Villareal et al 1997). Differences in GT may contribute to the closer amylopectin molecules in the cooked intermediate-GT high-AC starches, resulting in greater amylopectin staling relative to cooked low-GT starches. The relative shelf life of cooked rices differing in amylose and amylopectin staling is under investigation.

The development of a gel-consistency-type screening method for amylopectin staling to complement Instron hardness may utilize the waxy rices, Malagkit Sungsong and Tapol as reference amylopectins, starting with neutral gel consistency (Antonio & Juliano 1974, Juliano et al 1974, Antonio et al 1975, Perdon & Juliano 1975), the minimum KOH needed to disperse the waxy flour can be explored to minimize salt formation upon neutralization with acetic acid.

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